PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 April 2000 (12.04.00)	
International application No. PCT/US99/18869	**Applicant's or agent's file reference** 03063-0440WP
International filing date (day/month/year) 19 August 1999 (19.08.99)	**Priority date** (day/month/year) 21 August 1998 (21.08.98)
Applicant LAL, Altaf, A. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

16 March 2000 (16.03.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Diana Nissen
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

09/763397

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
03063-0440WP		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/18869	19/08/1999	21/08/1998
International Patent Classification (IPC) or national classification and IPC C12N15/30		
Applicant THE GOVERNMENT OF THE UNITED STATES OF ... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which hav been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16/03/2000	20.10.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Paresce, D Telephone No. +49 89 2399 8995

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/18869

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-31 as originally filed

Claims, No.:

1-10 as originally filed

Drawings, sheets:

1/5-5/5 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 7.

because:

☒ the said international application, or the said claims Nos. 7 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4
	No:	Claims	1-3, 5-10
Inventive step (IS)	Yes:	Claims	
	No:	Claims	4
Industrial applicability (IA)	Yes:	Claims	1-6, 8-10
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claim 7 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).

For the assessment of the present claim 7 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1) The documents mentioned in this communication are numbered as in the search report, i.e. D1 corresponds to the first document of the search report.

2) Novelty: Article 33(2) PCT

The subject-matter of claims 1-3, 5-10 is not considered new in the sense of Article 33(2) PCT for the following reasons:

D1 discloses recombinant protein particles carrying one or more epitopes that are produced using the pI protein of the retrotransposon Ty1 of S. *cerevisiae*. Recombinant Ty virus-like particles (TY-VLPs) were engineered which carried a string of up to 15 defined cytotoxic T lymphocyte (CTL) epitopes from *Plasmodium falciparum*, along with three T-helper epitopes, an antibody epitope and a heparin-binding adhesion motif. The Ty-VLPs have been used to create a candidate

vaccine for *Plasmodium falciparum* malaria. The recombinant Ty-VLPs were found to prime protective CTL responses in mice following a single administration without adjuvant (see D1, abstract and introduction, p. 1280). Table 1 of D1 shows the sequences included in the Ty-VLPs. In particular, D1 discloses the presence of is6 and is8 (corresponding to LSA, SEQ ID NO 9 and 10 of the present application) from the liver stage in the life cycle of *Plasmodium falciparum*, TRAP-AM (SSP-2, SEQ ID NO 8) from the blood stage and NANP (CSP, SEQ ID NO 4) from the sporozoite stage in one recombinant molecule. Figure 4 of D1 shows the recognition of Ty-VLPs with an anti-NANP monoclonal antibody. Figure 2 shows the induction of pb6-specific CTL in mice following a single immunization with Ty-VLPs.

The IPEA is of the opinion that the recombinant Ty-VLPs carrying CTL epitopes from *Plasmodium falciparum*, disclosed in D1, would fall under the scope of claims 1-3, 5-10 of the present application.

The subject-matter of claim 4 has not been made available to the public by any of the available prior art documents and can therefore be regarded as novel.

2) **Inventive Step: Article 33(3) PCT**

The subject-matter of claim 4 is not considered to involve an inventive step in the sense of Article 33(3) PCT for the following reasons:

In claim 4, a slight constructional change in the recombinant protein of claim 1 is defined which comes within the scope of the customary practice followed by persons skilled in the art, especially as the advantages thus achieved can readily be foreseen. The procedure of adding a signal peptide and polyhistidine to a recombinant protein has been described in the prior art (see D3) and is considered one of several straightforward possibilities from which the skilled person would select. The subject-matter of claim 4, therefore, does not involve an inventive step (Article 33(3) PCT).

VIII. Certain observations on the international application

1) **Clarity: Article 6 PCT**

Article 6 PCT requires amongst other things that the claims, which define the matter for which protection is sought (i.e. the object of invention) be clear. This has to be interpreted as meaning not only that a claim from a technical point of view must be comprehensible, but also that it must define clearly the object of the invention, that is to say, it must indicate all the essential features thereof. The essential features are regarded as all features which are necessary to obtain the desired effect, or differently expressed, those features which are necessary to solve the technical problem with which the application is concerned. In other words, all technical features which enable the skilled person to put the claimed matter into practice without undue burden i.e. without experimentation or without application of inventive skill.

In the present case, claims 2, 3, 6 are directed to a recombinant protein comprising peptides of given sequences or to "fragments, combinations, and conservative substitutions thereof". These terms should be more precisely defined. It is not clear if the claimed fragments or fragment combinations has the same properties as the peptide to which it refers. Without a definition of the length of the fragment or a precise definition of the meant part of the sequence in question, these claims are considered unclear. Furthermore, claim 2 is not clear because the term, "combinations thereof" does not clearly and unambiguously define the scope of the claim. Without a definition of how exactly the peptides or sequences are to be "combined", this phrase is absolutely vague and ambiguous.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
03063-0440WP		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/ 18869	19/08/1999	21/08/1998
Applicant		
THE GOVERNMENT OF THE UNITED STATES OF AM... et al		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNA AL SEARCH REPORT

International application No.
PCT/US 99/ 18869

Box I Observations where certain claims were found unsearchabl (Continuation of item 1 of first she t)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 7 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]T/US 99/18869

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/30 C12N15/62 C07K14/445 C07K16/20 A61K39/015
A61P33/06 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K A61P G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GILBERT ET AL.: "A protein particle vaccine containing multiple malaria epitopes" NATURE BIOTECHNOLOGY, vol. 15, November 1997 (1997-11), pages 1280-1284, XP002128163 page 1280, column 2 -page 1283, column 1; figures 1-4; table 1 ---	1-10
A	US 5 766 597 A (DE TAISNE CHARLES ET AL) 16 June 1998 (1998-06-16) column 4, line 8 - line 44 column 12, line 26 -column 13, line 14; claims 1-8; examples 35-39,47 --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 January 2000	04/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van Klompenburg, W

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]T/US 99/18869

C.(Continuation) DOCUMENTS CONS[redacted]RED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 33736 A (AFFYMAX TECH NV ;BARUCH DROR I (US); PASLOSKE BRITTAN L (US); HOWA) 31 October 1996 (1996-10-31) page 28, line 3 - line 10 page 74, line 1 - line 9	4
P,X	SHI ET AL.: "Immunogenicity and in vitro protective efficacy of a recombinant multistage Plasmodium falciparum candidate vaccine" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 96, no. 4, 16 February 1999 (1999-02-16), pages 1615-1620, XP002128164 cited in the application the whole document	1-10

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/18869

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5766597 A	16-06-1998	US 5494807 A	27-02-1996
		US 5756101 A	26-05-1998
		US 5364773 A	15-11-1994
		US 5453364 A	26-09-1995
		AU 701825 B	04-02-1999
		AU 7060294 A	03-01-1995
		CA 2168124 A	22-12-1994
		EP 0717636 A	26-06-1996
		WO 9428930 A	22-12-1994
		US 5759552 A	02-06-1998
		US 5756102 A	26-05-1998
		US 5863542 A	26-01-1999
		US 5756103 A	26-05-1998
		US 5766599 A	16-06-1998
		US 5989561 A	23-11-1999
		US 5759553 A	02-06-1998
		US 5744141 A	28-04-1998
		US 5843456 A	01-12-1998
		US 5762938 A	09-06-1998
		US 5503834 A	02-04-1996
		AU 701599 B	04-02-1999
		AU 1001197 A	15-05-1997
		AU 701781 B	04-02-1999
		AU 1001297 A	15-05-1997
		AU 672359 B	03-10-1996
		AU 1587192 A	06-10-1992
		AU 699903 B	17-12-1998
		AU 6051396 A	24-10-1996
		CA 2105277 A	08-09-1992
		EP 0575491 A	29-12-1993
		JP 6505874 T	07-07-1994
		WO 9215672 A	17-09-1996
		US 5833975 A	10-11-1998
		US 5641490 A	24-06-1997
		US 5766598 A	16-06-1998
		US 5942235 A	24-08-1999
		US 5759841 A	02-06-1995
		AU 674491 B	02-01-1997
		AU 1665792 A	21-10-1992
		AU 1668097 A	12-06-1997
		AU 709479 B	26-08-1999
		AU 1668197 A	12-06-1997
		CA 2106464 A	21-09-1992
		EP 0575544 A	29-12-1993
		JP 6505397 T	23-06-1994
		WO 9216616 A	01-10-1994
		US 5514375 A	07-05-1996
WO 9633736 A	31-10-1996	AU 5851296 A	18-11-1996

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
MCDONALD, John K.
JONES & ASKEW, LLP
2400 Monarch Tower
3424 Peachtree Road, N.E.
Atlanta, GA 30326
ETATS-UNIS D'AMERIQUE

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	21.06.2000
Applicant's or agent's file reference: 03063-0440WP	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/18869	19/08/1999	21/08/1998

International Patent Classification (IPC) or both national classification and IPC
C12N15/30

Applicant
THE GOVERNMENT OF THE UNITED STATES OF ... et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 21/12/2000.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner
Paresc , D

Formalities officer (incl. extension of time limits)
Gazzoli, M
Telephone No. +49 89 2399 2815

I. Basis f th pini n

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-31 as originally filed

Claims, No.:

1-10 as originally filed

Drawings, sheets:

1/5-5/5 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims 1-3, 5-10
Inventive step (IS)	Claims 4
Industrial applicability (IA)	Claims

2. Citations and explanations

s e separat sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claim 7 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).

For the assessment of the present claim 7 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1) The documents mentioned in this communication are numbered as in the search report, i.e. D1 corresponds to the first document of the search report.

2) Novelty: Article 33(2) PCT

The subject-matter of claims 1-3, 5-10 is not considered new in the sense of Article 33(2) PCT for the following reasons:

D1 discloses recombinant protein particles carrying one or more epitopes that are produced using the pl protein of the retrotransposon Ty1 of S. *cerevisiae*. Recombinant Ty virus-like particles (TY-VLPs) were engineered which carried a string of up to 15 defined cytotoxic T lymphocyte (CTL) epitopes from *Plasmodium falciparum*, along with three T-helper epitopes, an antibody epitope and a heparin-binding adhesion motif. The Ty-VLPs have been used to create a candidate

vaccine for *Plasmodium falciparum* malaria. The recombinant Ty-VLPs were found to prime protective CTL responses in mice following a single administration without adjuvant (see D1, abstract and introduction, p. 1280). Table 1 of D1 shows the sequences included in the Ty-VLPs. In particular, D1 discloses the presence of is6 and is8 (corresponding to LSA, SEQ ID NO 9 and 10 of the present application) from the liver stage in the life cycle of *Plasmodium falciparum*, TRAP-AM (SSP-2, SEQ ID NO 8) from the blood stage and NANP (CSP, SEQ ID NO 4) from the sporozoite stage in one recombinant molecule. Figure 4 of D1 shows the recognition of Ty-VLPs with an anti-NANP monoclonal antibody. Figure 2 shows the induction of pb6-specific CTL in mice following a single immunization with Ty-VLPs.

The IPEA is of the opinion that the recombinant Ty-VLPs carrying CTL epitopes from *Plasmodium falciparum*, disclosed in D1, would fall under the scope of claims 1-3, 5-10 of the present application.

2) **Inventive Step: Article 33(3) PCT**

The subject-matter of claim 4 is not considered to involve an inventive step in the sense of Article 33(3) PCT for the following reasons:

In claim 4, a slight constructional change in the recombinant protein of claim 1 is defined which comes within the scope of the customary practice followed by persons skilled in the art, especially as the advantages thus achieved can readily be foreseen. The procedure of adding a signal peptide and polyhistidine to a recombinant protein has been described in the prior art (see D3) and is considered one of several straightforward possibilities from which the skilled person would select. The subject-matter of claim 4, therefore, does not involve an inventive step (Article 33(3) PCT).

VIII. Certain observations on the international application

1) **Clarity: Article 6 PCT**

Article 6 PCT requires amongst other things that the claims, which define the

matter for which protection is sought (i.e. the object of invention) be clear. This has to be interpreted as meaning not only that a claim from a technical point of view must be comprehensible, but also that it must define clearly the object of the invention, that is to say, it must indicate all the essential features thereof. The essential features are regarded as all features which are necessary to obtain the desired effect, or differently expressed, those features which are necessary to solve the technical problem with which the application is concerned. In other words, all technical features which enable the skilled person to put the claimed matter into practice without undue burden i.e. without experimentation or without application of inventive skill.

In the present case, claims 2, 3, 6 are directed to a recombinant protein comprising peptides of given sequences or to "fragments, combinations, and conservative substitutions thereof". These terms should be more precisely defined. It is not clear if the claimed fragments or fragment combinations has the same properties as the peptide to which it refers. Without a definition of the length of the fragment or a precise definition of the meant part of the sequence in question, these claims are considered unclear. Furthermore, claim 2 is not clear because the term, "combinations thereof" does not clearly and unambiguously define the scope of the claim. Without a definition of how exactly the peptides or sequences are to be "combined", this phrase is absolutely vague and ambiguous.

2) Additional comments:

Should the applicant file a new set of claims, which take account of the above comments, he is requested to clearly identify the amendments carried out, no matter whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based (see Rule 66.8(a) PCT), to facilitate the examination of the conformity of the amended application with the requirements of Article 34(2)(b) PCT. If the applicant regards it as appropriate these indications could be submitted in handwritten form on a copy of the relevant parts of the application as filed. The attention of the Applicant is drawn to the fact that the application may not be amended in such a way that it contains subject-matter which extends beyond the content of the application as filed, Article 34(2)(b) PCT.

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A Population-Based Clinical Trial with the SPf66 Synthetic *Plasmodium falciparum* Malaria Vaccine in Venezuela

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A phase III malaria vaccine trial in 13 villages in an endemic area, South Venezuela, compared incidence rates of *Plasmodium falciparum* and *Plasmodium vivax* infections in 1422 vaccinated and 938 nonvaccinated subjects over 18 months. The SPf66 vaccine was given in three doses, on days 0, 20, and 112. Vaccination was complete in 976 subjects (68.7%). Minor side effects requiring no treatment were reported by 123 (12.6%), with an apparent increase in frequency from the first to the third vaccine dose. No autoimmune evidence was observed in a sample of subjects. Antibodies against SPf66 were present at low titers in 24.7% of tested subjects before vaccination, increasing to 53.6% after the second dose and to 73.6% after the third dose; 26.4% of subjects initially seronegative never seroconverted. The SPf66 malaria vaccine showed a protective efficacy of 55% (95% confidence interval, 21%–75%) against *P. falciparum* and of 31% (10%–47%) against *P. vivax* malaria.

The operational difficulties and the limitations of the available tools for malaria control, further compromised by the progressive resistance of parasites to drugs and of vectors to insecticides, have given impetus to explore new strategies for the prevention of malaria transmission.

A remarkable effort has been placed on malaria vaccine development. Since the first attempts to immunize humans with irradiated sporozoites [1], several different approaches have been pursued, targeting different parasite stages and antigens, either chemically synthesized or developed by genetic engineering. As a result, several parasite proteins or fragments have been characterized, some have been shown to confer protective immunity in animal models [2, 3] and human studies [4–7]. Peptidic and recombinant candidate subunit vaccines based on the *Plasmodium falciparum* circumsporozoite protein have been tested in challenge and field studies [3, 7]. Although these vaccines were found to be safe and to induce antibody responses, they failed to demonstrate a solid protective efficacy.

The SPf66 hybrid malaria vaccine, developed in Colombia, is a polymeric synthetic peptide containing amino acid sequences of three *P. falciparum* antigenic proteins linked by the Asn-Ala-Asn-Pro tetrapeptide from the circumsporozoite [4]. This vaccine evokes immune responses against the asexual stages of the erythrocytic cycle of *P. falciparum* and had been shown to be safe and immunogenic in human trials [3, 8, 9]. Clinical field studies carried out after the trial reported here have demonstrated the expected evidence, showing a 40%–82% protective efficacy against *P. falciparum* in certain defined age groups [10–12].

Here we report the results of the first population-based phase III trial with the SPf66 synthetic malaria vaccine conducted in an endemic area.

Materials and Methods

Study area and population. The study was carried out in 13 small villages, covering a population of 2,360 inhabitants in the municipality of Las Majadas, Piar District, Bolívar State, South Venezuela. The climate is typical of a rainy tropical savanna, with a mean annual temperature of 26–28°C, average annual rainfall of ~2000 mm, an altitude of 50 m above sea level, and wind velocity of 4 km/h. A wet season extends from May to October and a dry season from November to April [13]. The peak of malaria transmission occurs during the months of December through March. According to the National Malaria Control Program, the mean annual incidence of *P. falciparum* malaria for

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Informed consent was obtained from subjects and the procedures were approved by an Ethical Committee representing the Ministry of Health, Universidad Central de Venezuela, and Universidad de los Andes.

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this region during the 5 years before the trial was 100/1000 populati n. *P. falciparum* malaria represented ~25% of the cases reported. Asymptomatic parasitemia is seldom observed. n r is resistance t chloroquine chemotherapy. The main malaria vectors identified in the area were *Anopheles darlingi*, *Anopheles oswaldoi*, and *Anopheles albitarsis*.

The eligible population for the study comprised all persons >11 years old living in the study area (n = 2439). A population census was done through household visits recording age, sex, occupation, literacy, religion, pregnancy, length of residence in the area, mobilization habits, type of dwelling, use of antimalarial drugs in the last 2 months, use of antimosquito nets, and medical history from all household members. The census was updated during the study by three cross-sectional observations to derive person-time of exposure among the participants and to collect blood and urine samples for immune response and antimalarial self-medication tests.

Vaccination. The SPf66 vaccine was synthesized at the Instituto de Inmunologia, Hospital San Juan de Dios, Bogotá, Colombia, following the solid-phase peptide synthesis method described by Merrifield [14] as modified by Houghten [15]. Each dose consisted of a volume of 0.5 mL containing 4 mg/mL SPf66 peptide adsorbed onto alum hydroxide at a 2 mg/mL concentration. Details of the formulation, quality control assays, and preservation have been described elsewhere [4].

Before vaccination, community meetings were organized to explain the possible benefits and risks of the vaccine and the v luntary nature of he vaccination. All persons residing in the community who spontaneously attended an open invitation for vaccination were vaccinated (n = 1422). The vaccine was administered subcutaneously in the deltoid, in three doses, on days 0, 20, and 112. Because of logistics, 160 subjects received the third dose on day 156. After each dose, the participants were observed by a clinician over 45 min and were advised to contact the local health service during the following 48 h in case of any abnormal clinical manifestation. The recruitment and vaccination lasted ~4 days for each dose. All volunteers were clinically examined, and the following were excluded from vaccination: pregnant women; persons with severe health problems, mental disorders, alcoholism, or history of allergies or debilitating diseases; those receiving immunosuppressive drugs; and those with clinical symptoms suggestive of malaria. Subjects eligible for vaccination were examined by thick and thin blood smear 15 days before vaccination. Malaria cases were treated according to the National Malaria Control Program; therefore, by the time of vaccination, only persons asymptomatic and free of parasitemia were vaccinated. Pregnancy was ascertained by interview and also by a urine test (Pregna P test, Precisa, Miami) before each vaccine dose in all women >15 years of age.

After the three doses of vaccine were given, a comparison group of 938 residents included in the initial census who did not attend the vaccination day was selected; persons who had any of the exclusion criteria applied to the vaccination group were excluded.

The study sample size has an 80% power, at a 5% significance level, to detect a 50% reducti n in malaria rate over a period of 1 year, assuming an expected annual *P. falciparum* malaria incidence in the unvaccinated group of 3.9%, as reported in the previ us year.

Follow-up observations. The follow-up bservation for vaccinated and nonvaccinated subjects extended over 14 months after the third vaccine dose. Household visits were carried out by health field workers every 2 weeks, who asked for clinical symptoms of malaria throughout the study. A blood slide was prepared for all symptomatic subjects. Also, active case-finding detection was implemented by taking thick and thin blood smears from all participants 15 days before vaccination and every 8–10 weeks after the second dose of vaccine. Passive case detection and malaria treatment as prescribed by the National Malaria Control Program was provided by a local health facility attended by rural physicians assigned exclusively to the study.

A malaria case was defined as presence of malaria parasites in the circulating blood. A new *P. falciparum* malaria episode was defined as malaria parasites in a blood smear after a period of at least 30 days free of parasitemia. All slides were examined in the field by trained personnel and then double-checked blindly at the Central Laboratory of the National Malaria Control Program and at the Laboratory for the Study of Malaria, Ministry of Health/Universidad Central de Venezuela (UCV).

Self-medication with antimalarial drugs was ascertained by interview and also by random testing of urine samples from 289 vaccinated and 180 nonvaccinated subjects. Samples were collected 6 and 12 months after the third dose and tested by thin-layer chromatography [16] to detect 4-aminoquinoline excretion. All samples were tested at UCV and at the Swiss Institute for Tropical Medicine (Basel).

Malaria chemoprophylaxis, treatment of the patient's family, presumptive treatment, and nebulization with insecticide were all suspended during the study period. Insecticide spraying with DDT every 6 months was the only malaria control intervention maintained.

Immune response. Antibody tests against native *P. falciparum* proteins were done before vaccination by indirect immunofluorescence assay (IFA). Titers ≥1:80 were considered positive. Paired blood samples collected on the first day of vaccination, 30 days after the second dose, and 1, 6, and 12 months after the third dose were stored at −80°C and processed simultaneously at the end of the study. Antibodies to SPf66 polymer were titered by ELISA as described [17]. A cutoff point was established as the mean optical density + 3 SD of the results obtained testing a large number of serum samples from persons who had never lived in an endemic area.

Autoimmune responses were also evaluated by testing serum samples collected from a random sample of participants before vaccination and 1 month after completing the third dose. Tests included determination of IgG, IgA, IgM, and C3 and C4 complement components (Kallestad QM 300 protein analysis system; Sanofi Diagnostics Pasteur, Chaska, MN) by nephelometry [18]; complement B factor by radial immunodiffusion [19]; IgE by RIA (Quanticlone IgE Kit; Sanofi) [20]; antinuclear antibodies by immunofluorescence (Quantafluor substrate slides Hep.2; Sanofi); circulating immune complexes by ELISA [21]; and rheumatoid factor by nephelometry [18].

Statistical analysis. Baseline characteristics of vaccinated and unvaccinated subjects were compared. Mean age, sex distribution, and proportions of participants with the different characteristics, including antibody response, were calculated. Differ-

ences between proportions were evaluated by χ^2 and differences between means by Student's t test [22]. A significance level of .05 was used.

P. falciparum and *Plasmodium vivax* malaria incidences were calculated monthly, taking persons at the end of the study and also person-time of follow-up in each group as denominators. Incidence rates were examined according to age, sex, ethnic group, occupation, place of residence, and malaria history. To compare the incidence of malaria after vaccination between the vaccinated and unvaccinated groups adjusting for a dissimilar malaria risk at baseline, rate ratios were calculated for the incidence during the 12 months subsequent to the third dose of vaccination in relation to that observed during an equivalent calendar period just before vaccination, separately for *P. falciparum* and *P. vivax*. The after- to before-vaccination incidence ratio of each group was used to derive vaccine efficacy (VE) as $VE = 1 - (R_v/R_{nv})$, where R_v and R_{nv} are ratios of the incidence after vaccination to the incidence before vaccination for the vaccinated and nonvaccinated groups, respectively [23]. χ^2 significance tests and 95% confidence intervals of the estimates were calculated. This procedure was required to have a proper assessment of the effect of immunization on malaria incidence taking into account that the two groups were not randomized to be vaccinated and presented with different characteristics of risk of infection.

Results

Of the 1422 subjects who received the first vaccine dose, 976 (68.7%) completed the full three-dose vaccination schedule, 362 (25.5%) received two doses, and 84 (5.9%) received just the first vaccine dose. Noncompliance with vaccination was attributed to absence from the community on the day of vaccination, pregnancy, and other exclusion criteria. No statistical difference was observed regarding age, sex, or occupation distributions between the vaccinated and comparison groups. Mean age of participants was 32.7 years, and ~58% were male. Occupations with a high risk of exposure to infection, such as farmer, fisher, hunter, and miner, were reported by 55% of controls and 51% of vaccinated subjects. Most participants (79.7%) were mestizos. The proportion of subjects identified as Amerindian was greater among those not vaccinated than among vaccinated subjects (14.9% vs. 6.9%; $P < .01$).

Persons receiving vaccination lived predominantly in localities of higher risk of transmission than nonvaccinated subjects. However, the history of malaria as reported during the interviews was similar for both study groups. Also, the proportions of subjects reporting use of antimalarial drugs during the follow-up period did not differ statistically between the two groups (10% vs. 12%). Urine tests for aminoquinolines were positive in 13.8% of nonvaccinated and in 11.7% of vaccinated subjects ($P > .05$). Analysis of records from the National Malaria Control Program indicated that annual *P. falciparum* malaria incidences during the 5 years before the study increased progressively from 0.6% to 6.3% among unvaccinated subjects and from 0.8% to 8.3% among vaccinees. Corresponding rates for *P. vivax* malaria increased from 1.5% to 11.3% for controls and from 0.9% to 20.4% among vaccinees.

Table 1. Side effects of SPf66 vaccine according to number of doses

Side effect	Dose 1 (n = 1420)	Dose 2 (n = 1338)	Dose 3 (n = 976)
Induration	0.3	0.3	5.6
Pain	0.7	0.5	0.4
Erythema	0.1	0.4	0.5
Local pruritus	0.1	0.8	3.5
Generalized pruritus	0	0.1	0.5
Bronchospasm	0	0	0.2
Other	0	0.3	0.3

NOTE. Data are % with side effect.

Side effects. Minor side effects requiring no treatment were reported in a small proportion of subjects (table 1). Local pain, induration, and pruritus were the most common reactions observed. Contralateral induration and local erythema were more frequent after the third dose. Five women presented with systemic pruritus outside of the injection site, and 1 case of bronchospasm was reported, occurring 15 min after vaccination in an apparently healthy woman. The frequency of systemic reactions was significantly higher in women. No case of delayed reaction was observed. No generalized reaction was reported for subjects receiving one or two vaccine doses.

Autoimmune tests. No abnormalities were seen in the comparison of results of the various autoimmunity serum marker tests done before vaccination and 30 days after application of the third dose in a sample of 43 vaccinated subjects, suggesting that the vaccine was safe in this respect. None of the immunologic parameters analyzed showed statistically significant variation. In 1 vaccinated subject, the level of C3 and C4 decreased, while values for factor B and circulant immune complexes were normal.

Immunogenicity. The prevalence of native fluorescent antibodies to *P. falciparum* before vaccination was 54.9% for the vaccine group and 51.9% for the nonvaccinated subjects ($P < .05$). No significant change in these prevalences was observed after vaccination. Of the 929 tested vaccinated subjects, 24.7% had anti-SPf66 antibodies at low titers (1:100–1:400) before vaccination. Sixty days after the second vaccine dose, 53.6% of the study population showed anti-SPf66 antibodies, 76.6% of samples with titers of <1:400. Thirty days after completing the three-dose vaccination, 60.4% of subjects were positive to antibodies against the peptide. In this group, 552 (80.9%) were classified as low responders, with titers of <1:400.

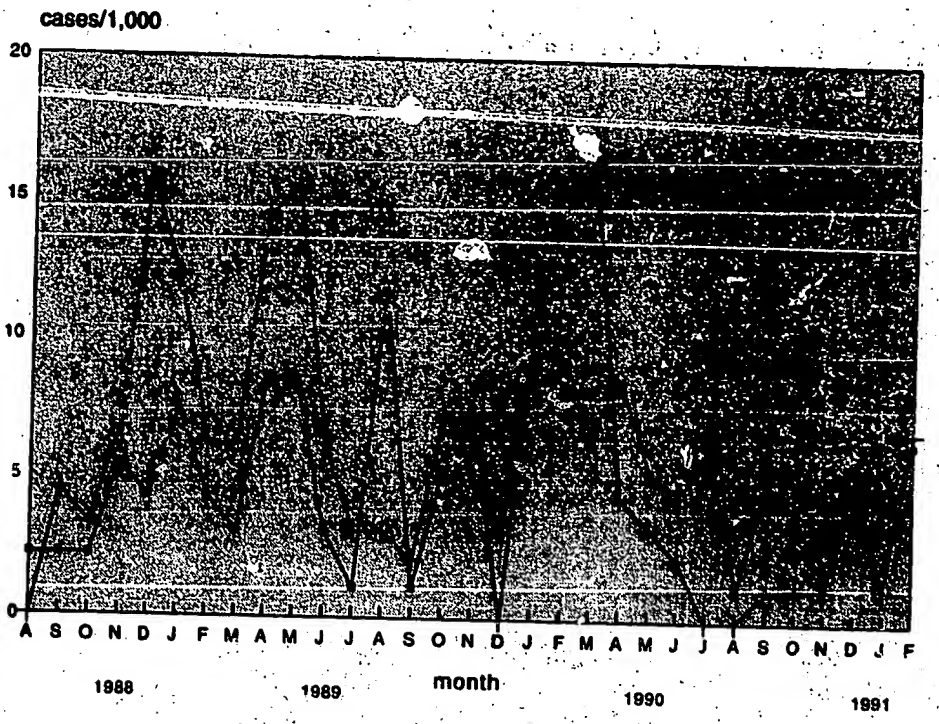

Figure 1. Monthly incidence of *P. falciparum* malaria among vaccinated and unvaccinated persons, covering 1 year before vaccination up to end of study, Las Majadas, 1988–1991.

Of subjects initially negative for antibodies to SPf66, 26.4% never seroconverted and 48.4% seroconverted after the second and third doses, with titers falling to <1:100 by the end of follow-up. One year after the third dose, 25.2% of those showing seroconversion still had positive titers.

Protective efficacy. Figure 1 shows monthly *P. falciparum* malaria incidences for both study groups. There was a significant decrease in incidence starting 3 months after the third dose of vaccine. During the 12 months immediately after the third dose, the incidence of *P. falciparum* malaria was 4.5/1000 person-months at risk for the vaccinated group and 5.6/1000 person-months for the nonvaccinated group. Protective efficacy conferred by the vaccine was calculated by taking the ratio of the incidence during the follow-up period to the baseline incidence for the same amount of time before vaccination as a way to control for seasonal variation in incidence. The vaccinated group had a mean incidence before vaccination of 7.9/1000 person-months, while the nonvaccinated group had a mean incidence of 4.4/1000 person-months (table 2). On the basis of these estimates, the vaccine protective efficacy for those receiving the complete series of three doses was calculated to be 55.1% (95% confidence interval, 21%–75%; $P < .01$). Protective efficacy calculated for subjects receiving only two doses of vaccine was ~67%, with wide and nonsignificant confidence intervals because of the small numbers of subjects and cases in this group.

The incidence of *P. falciparum* malaria was higher, but not statistically significant, for subjects who did not seroconvert after immunization (3.8%) than for those who seroconverted (1.3%). Among nonvaccinated subjects, the incidence of malaria did not differ between those negative and positive for native SPf66 antibodies.

Monthly *P. vivax* malaria incidences covering 1 year before vaccination and the follow-up period are shown in figure 2. Conversely to *P. falciparum* malaria, incidences of *P. vivax* increased after vaccination in both groups, mostly in the last quarter of the follow-up year. For the vaccinated group, the incidences during the 12 months preceding and following vaccination were 16.3/1000 and 30.7/1000 person-months, respectively. Corresponding figures for the nonvaccinated group were 9.1/1000 and 28.9/1000 person-months (table 3). The vaccine's protective effect against *P. vivax* malaria was estimated as 41% (95% confidence interval, 19%–57%). No further adjustment to estimate vaccine efficacy was considered necessary, since incidences were compared within the same group of subjects before and after vaccination.

Table 2. Cumulative incidence of *P. falciparum* malaria during 12 months preceding and following the complete series of three doses of vaccine.

	Vaccine	Control
Before vaccination		
Cases/person-months	89/11,289	49/11,171
Rate/1000 person-months	7.9	4.4
After vaccination		
Cases/person-months	46/10,223	56/9904
Rate/1000 person-months	4.5	5.6

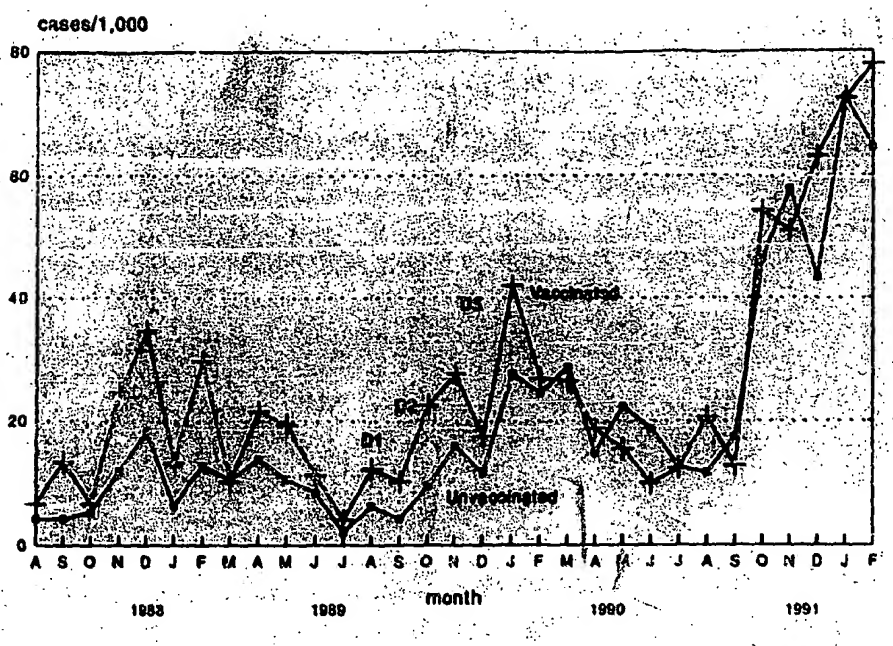

Figure 2. Monthly incidence of *Plasmodium vivax* malaria among vaccinated and unvaccinated persons, covering 1 year before vaccination up to end of study, Las Majadas, 1988–1991.

Discussion

The SPf66 vaccine was evaluated in Venezuela after promising results were obtained in initial preclinical studies in *Aotus* monkeys [3, 24] as well as in hospital and field clinical studies among Colombian military personnel [25]. The Las Majadas trial was initiated in August 1989 and corresponded to the first population trial with a synthetic malaria vaccine in a civilian population from an endemic area.

This trial was proposed with the objective of further expanding the study of safety, immunogenicity, and protection of the SPf66 vaccine in an area geographically distant from those originally studied in Colombia [10], with a different ethnic population and parasite strains and with a higher proportion of *P. vivax* infections. For ethical reasons, it was designed as an open trial, because it was thought that vaccination could delay the usual early health care–seeking behavior of symptomatic persons in this area.

Full compliance with vaccination was achieved by 68.7% of participants. Migration out of the study area accounted for the majority of persons lost during follow-up, occurring in a similar proportion among the vaccinated and nonvaccinated groups (31.1% and 33.9%).

Table 3. Cumulative incidence of *Plasmodium vivax* malaria during 12 months preceding and following the complete series of three doses of vaccine.

	Vaccine	Control
Before vaccination		
Cases/person-months	184/11,289	102/11,171
Rate/1000 person-months	16.3	9.1
After vaccination		
Cases/person-months	314/10,223	286/9904
Rate/1000 person-months	30.7	28.9

Although the vaccinees were not randomly selected, no statistical difference was observed regarding personal characteristics, history of malaria, consumption of antimalarial drugs during follow-up, or prevalence of prevaccination antibodies to the SPf66 polymer and to *P. falciparum* antigens. However, vaccinees lived in areas of higher malaria incidences than the controls and consequently had significantly higher *P. falciparum* and *P. vivax* malaria incidences during the year before vaccination.

The vaccine was very well tolerated. Side effects were rare and mostly at the site of injection. The effects reported were similar to those observed with other multiple-dose vaccines and with alum hydroxide as adjuvant [26]. The frequency of postvaccination effects other than local reactions was significantly higher in women (0.52% vs. 0). Local side effects were less frequent than those reported in Ecuador [12] but higher than those reported in Colombia [10, 11].

The autoimmunity serum markers studied showed absence of vaccine-induced injury. Just 1 subject showed a minor alteration of one immunologic parameter 30 days after application of the third dose. Chemical, hematologic, and autoimmune profiles reported in other studies also demonstrated that the vaccine is considered to be innocuous [4, 8, 9, 24].

Baseline immune response to *P. falciparum* was high: 24.7% of vaccinees and 28.5% of nonvaccinees recognized the SPf66 polymer, and 54.9% and 51.9%, respectively, were also positive by IFA, indicating a similar prevaccination humoral immune response in both groups. The higher sero-

prevalence of *P. falciparum* antibodies in relation to that found in other studies [11, 12] could be associated with a higher exposure to *P. vivax* and *P. falciparum* infections in this population. Of vaccinees initially negative, 26.4% remained seronegative throughout the study. However, most of the responders had low titers. Possible genetic differences could account for this finding, since the HLA-DR4 antigen carrier state has been reported to be associated with a low or nonexistent humoral immune response to the peptide [27, 28].

This study confirms previous reports showing no correlation between seroconversion and risk of *P. falciparum* infection [4], suggesting that ELISA cannot discriminate between protective and nonprotective antibodies. Markers of protective immune response induced by the vaccine should be investigated. Further studies are planned to correlate specific humoral immune responses to side effects and vaccine immunogenicity to prevaccination antibody levels.

To adjust for dissimilar malaria risks in vaccinated and nonvaccinated groups, protective efficacy was estimated by comparing changes in malaria incidence rates before and after vaccination between the two groups. The protective efficacy for *P. falciparum* infection for subjects receiving three doses was 55.1%. This is an intermediate value between results obtained in La Tola, Colombia (33.6%) [11], and La T, Ecuador (66.8%; [12].

In contrast to what was observed for *P. falciparum*, incidences for *P. vivax* increased in both groups after vaccination, particularly among the nonvaccinated group. This was reflected in a crude protective efficacy of 41% with a wide confidence interval. This unexpected result deserves special attention because this was the first SPf66 trial conducted in an area with a high *P. vivax* incidence rate, allowing for proper statistical analysis. A theoretical biologic explanation for a possible cross-protective effect is the existence of common protein sequences in both the SPf66 peptide and relevant *P. vivax* protein [29, 30] (figure 3). Also, SPf66-vaccinated persons show recognition of *P. vivax* antigen by IFA [31]. In addition, if protective *P. falciparum* nonspecific immune responses are elicited by SPf66, it could also affect other malaria parasite species.

We cannot rule out all possible biases in the interpretation of the study results, considering some limitations of the study design. The follow-up observation was similar in the two study groups. It is unlikely that the precision of malaria diagnosis was different between vaccinees and controls, since all blood slides were read blindly in a central laboratory. The increased exposure to malaria infection among vaccinees was controlled in the statistical analysis by estimating within groups malaria incidence changes over time, before and after vaccination. A crude comparison of the groups could have underestimated the protective efficacy conferred by the vaccine. The consumption of antimalarial drugs assessed by interviews and validated by urine analysis of 4-aminoquinolines showed no differences between the two groups, excluding the possibility of increased ingestion among vaccinees after complete vaccination.

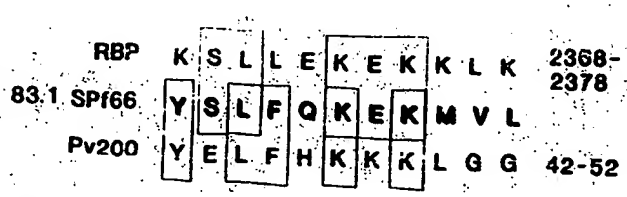

Figure 3. Comparison of amino acid sequences of SPf66 (83.1 peptide) and of known *Plasmodium vivax* protein molecule sequences, reticulocyte binding protein (RBP, top) and Pv200 (bottom). Amino acid residue numbers are given at right.

The development of an efficacious synthetic vaccine opens an exciting perspective of future investigation in this field for other infectious diseases. The synthetic nature of the vaccine allows the development of a more rational and simpler approach to further improve the former vaccine design.

This was the first field trial in a civilian population with a chemically synthesized vaccine and the first with a vaccine against malaria. In the present study, SPf66 was safe and immunogenic; under the characteristics of this trial, it conferred protection of ~55% against *P. falciparum* and 41% against *P. vivax* infections. Further studies should evaluate the impact of SPf66 in different ecoepidemiologic situations. The potential public health application of a malaria vaccine could substantially contribute to the global effort for the control of malaria.

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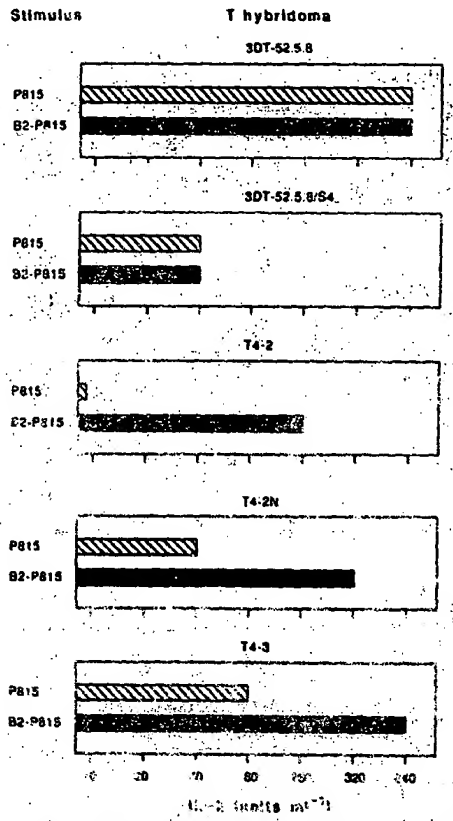

Fig. 3 Response of T-cell hybridomas to HLA-DR⁺ and untransfected P815 cells. T hybrid cells (10^5) were cocultured with 10^5 target cells at 37 °C, 5% CO_2 in 96-well culture plates (Flow); final volume of 200 µl in culture medium (modified Mishell-Dutton medium/10% FBS/5×10^{-5} M 2-mercaptoethanol/50 µg ml^{-1} gentamycin (Schering)). After 24 h, the culture supernatants were assayed for the presence of IL-2 by their ability to support the growth of the IL-2-dependent T-cell line HT-2 (ref. 25). The highest of 2-fold serial dilutions capable of maintaining 90% HT-2 cell viability defined the IL-2 concentration of the supernatant. Ten units ml^{-1} IL-2 was the minimum concentration noted. T-cell hybrids cultured in the absence of the appropriate antigen/MHC stimulus failed to produce any detectable IL-2 (data not shown).

ligand pairs may be a general phenomenon of cell recognition.

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Induction of protective immunity against experimental infection with malaria using synthetic peptides

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Synthetic peptides are potential vaccine candidates because they may be able to induce high antibody titres and specific cellular immune responses against native proteins and thus the whole invading organism[1]. In a previous study we showed that immunization with molecules of relative molecular mass (M_r) 155,000 (155K) 83K, 55K and 35K, specific for the late schizont and merozoite stages of *Plasmodium falciparum*, could elicit either partial or total protection in *Aotus trivirgatus* monkeys experimentally infected with *P. falciparum*[2]. Here we have chemically synthesized 18 peptides corresponding to different fragments of these proteins to immunize *Aotus trivirgatus* monkeys. Some peptides gave partial protection from challenge with *P. falciparum* parasites, but none provided complete protection individually. A combination of three partially protective peptides gave complete or almost complete protection, however, suggesting that this particular combination of peptides is a good candidate for a malaria vaccine.

The 155K, 83K, 55K and 35K proteins were isolated from schizont and merozoite lysates, in quantities ranging from 200–400 µg each, using preparative SDS-PAGE. These proteins showed a high degree of purity as seen by analytical SDS-PAGE and Western blots with hyperimmune sera from malaria patients. The 55K protein provided incomplete protection (defined as a significant delay in the onset of parasitaemia) and the 35K protein partial sterilizing protection (spontaneous control of the experimental infection without drug therapy) on challenge of immunized monkeys with *P. falciparum*[2].

We determined the sequences of the first 21 N-terminal amino acid residues of the 55K and 35K proteins. Based on these data and the amino acid sequences described for the 155K[3] or RESA molecule by Coppel *et al.*[4] together with the complete amino acid sequence of the 195K precursor of the 83K molecule[5], 18 peptides corresponding to different segments of the 35K, 55K, 83K and 155K proteins were synthesized using Multiple Solid Phase Synthesis[6]. These peptides were chosen randomly to represent different predicted conformations as determined by the Chou and Fasman method (Table 2), taking into account the suggestion that certain structures can preferentially induce humoral and cellular immune responses[7,8].

Groups of four to six Colombian *Aotus trivirgatus* monkeys were immunized with purified peptide (250 µg) coupled to BSA (250 µg) on days 0, 30, 45, 60 and 75. Blood samples for antibody studies were taken on days 50, 70 and 80. On day 90, 15 days after the last immunization, the monkeys were challenged with live *P. falciparum*.

Of the 18 different synthetic peptides used for immunization, 15 elicited antibodies against *P. falciparum* schizonts, as detected by immunofluorescence and were found to be immunogenic by

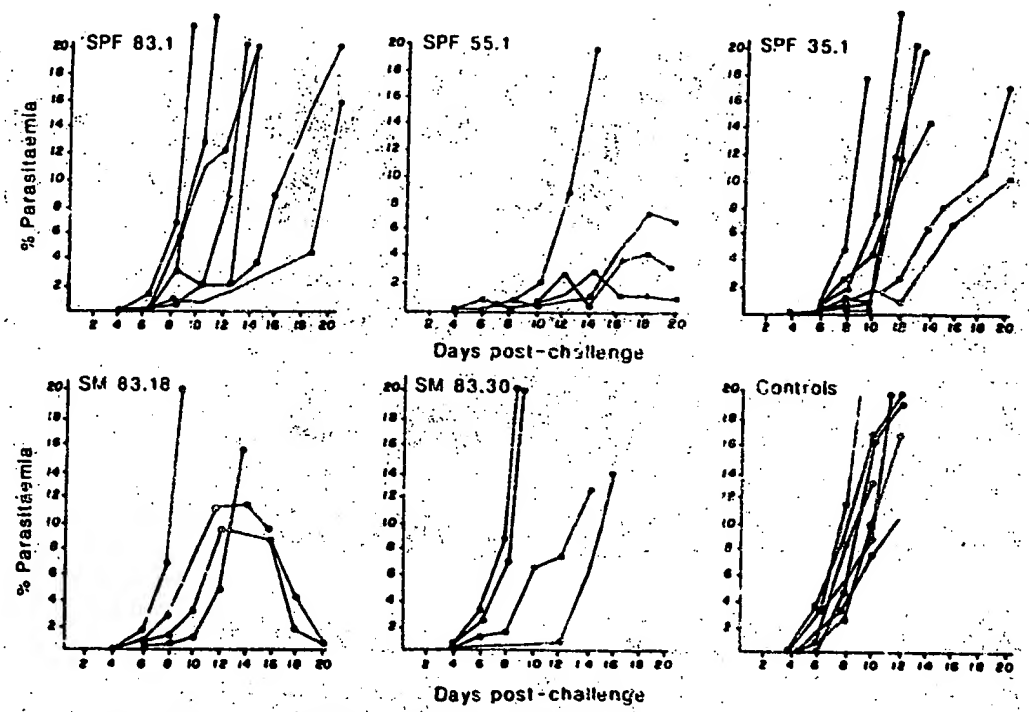

Fig. 1 Development of parasitaemia in monkeys immunized with peptides corresponding to different segments of the 83K, 55K and 35K proteins. Parasitaemia is expressed as the percentage of infected cells. Each line represents a different monkey.
Methods. Groups of 4-6 monkeys were immunized with a single peptide. Peptides (250 μg) had been previously coupled to an equal amount of BSA with glutaraldehyde in a total volume of 250 μl. Conjugated peptides were diluted 1:1 with complete or incomplete Freund's adjuvant (CFA or IFA) and used for subdermal immunization in several parts of the body. Controls received saline solution 1:1 in Freund's adjuvant. Immunizations were carried out on days 0 (with CFA), 30, 45, 60 and 75 (with IFA). The challenge was performed on day 90 with 5×10^6 live fresh *P. falciparum* parasites obtained from another *A. trivirgatus* monkey. This donor was heavily infected with the *P. falciparum* FVO strain (kindly provided by Dr Ruth Nussenzweig) adapted to grow in these monkeys in which it induces a lethal disease. Parasitaemia was monitored daily by examination of smears of fresh peripheral blood treated with the anticoagulant heparin, diluted 1:1 with phosphate buffered saline (PBS), and stained with Giemsa and/or acridine orange.

the dot-blot peptide-antipeptide assay[10]. The three peptides that did not elicit antibodies in this immunization scheme, SM 83.15, SM 83.29 and SM 83.23, are sequences from 83K molecule.

Most of the peptides tested failed to provide complete protection against experimental infection regardless of the presence of antibodies (data not shown). Some peptides, however, significantly delayed the onset of the disease in some of the vaccinated animals, suggesting an ability to induce incomplete protection. These peptides were SPf 45.1 and SPf 35.1 (corresponding to the N-terminal sequences of the 55K and 35K molecules respectively) and SPf 83.1, SPf 83.18 and SPf 83.30 (corresponding to fragments of the 83K protein) (Fig. 1). Some other peptides, for example SPf 83.2, SM 83.23 and SM 83.26 induced incomplete protection in one of four immunized monkeys. Studies are in progress to examine protection in a larger number of animals. None of the peptides used, however, provided complete protection (defined as total absence of parasites in blood smears).

We immunized a new group of monkeys with combinations of two or three of the partially protective synthetic peptides,

Table 1 Antibody titres of immunized monkeys

Sera from monkey no.	Immunized with peptide mixture	IIFA titre	Dot blot-ELISA with SPf 35.1	Dot blot-ELISA with SPf 55.1	Dot blot-ELISA with SPf 83.1
229	35.1+55.1	20	0	20	ND
255	35.1+55.1	80	20	160	ND
287	35.1+55.1	160	40	0	ND
251	35.1+55.1	160	10	20	ND
275	35.1+55.1	40	10	40	ND
288	35.1+55.1	5	10	40	ND
289	35.1+55.1	5	10	80	ND
286	35.1+55.1	160	10	40	ND
295	35.1+55.1+83.1	10	0	640	640
298	35.1+55.1+83.1	10	40	320	320
290	35.1+55.1+83.1	80	0	40	640
291	35.1+55.1+83.1	80	20	640	640
297	35.1+55.1+83.1	40	40	160	640
300	35.1+55.1+83.1	160	160	160	320

Antibody titres of monkeys immunized with the peptide mixtures were determined by indirect immunofluorescence assay (IIFA) and dot-blot ELISA and are expressed as the reciprocal of the serum dilution end-point. In IIFA, air-dried *P. falciparum* erythrocyte cultures with 5% parasitaemia at the schizont stage were used. For the dot-blot ELISA, peptide-antipeptide reactivity was determined using 2 μl of purified peptides at 3 mg ml^{-1} placed on nitrocellulose sheets and dried. Non-specific reactive sites were neutralized with Tris buffered saline solution (TBS) supplemented with 0.5% Tween 20. Sheets were covered with the appropriate serum dilution for 2 h, washed several times with Tween 20 TBS and incubated with alkaline phosphatase affinity purified anti-human IgG for 2 h. After five more washes with Tween 20 TBS, the reaction was developed with BCIP (5-bromo-4-chlor indolyl phosphate) and NBT (nitroblue tetrazolium), as described by Blake[12]. All the pre-immune and control sera were negative by both tests.

Table 2 Synthetic peptides used for immunization

Denomination	Residue number	Original molecule	Sequence
α-Helix			1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
SPf 35.1	N-terminal	35K	Tyr-Gly-Gly-Pro-Ala-Asn-Lys-Lys-Asn-Ala-Gly-OH
SPf 55.1	N-terminal	55K	Asn-Glu-Leu-Glu-Ala-Glu-Thr-Gln-Asn-Val-Tyr-Ala-Ala-NH_2
SPf 155.1	Repeated octapeptide	155K	Glu-Glu-Asn-Val-Glu-His-Asp-Ala-Tyr-NH_2
SPf 83.1	43 - 53	195K	Tyr-Ser-Leu-Phe-Gln-Lys-Glu-Lys-Met-Val-Leu-NH_2
SM 83.10	27 - 38	195K	Glu-Leu-Val-Lys-Lys-Phe-Glu-Ala-Leu-Glu-Asp-Ala-OH
SM 83.2	40 - 52	195K	Leu-Thr-Gly-Tyr-Ser-Leu-Phe-Gln-Lys-Glu-Lys-Leu-Val-OH
SM 83.26	640 - 652	195K	Leu-Glu-Lys-Leu-Thr-Lys-Ala-Leu-Lys-Tyr-Leu-Glu-Asp-OH
SM 83.28	575 - 587	195K	Glu-Asn-Glu-Ile-Glu-Thr-Leu-Val-Glu-Asn-Phe-Lys-Lys-OH
SM 83.29	584 - 595	195K	Asn-Ile-Lys-Lys-Asp-Gly-Glu-Gln-Leu-Phe-Glu-Lys-OH
SM 83.34	731 - 742	195K	Gln-Ala-Gln-Ala-Gln-Gln-Gln-Lys-Glu-Ala-Gln-Tyr-OH
β-Sheet			
SM 83.31	614 - 624	195K	Phe-Val-Lys-Val-Gln-Val-Gln-Lys-Val-Leu-Leu-OH
β-Turns			
SM 83.6	78 - 92	195K	Ser-Gly-Gly-Ser-Val-Ala-Ser-Gly-Gly-Ser-Val-Ala-Ser-Gly-Gly-OH
SPf 83.2	106 - 117	195K	Tyr-Asn-Ser-Arg-Arg-Thr-Asn-Pro-Ser-Asp-Asn-Gly-OH
SM 83.15	239 - 250	195K	Glu-Asp-Tyr-Ile-Lys-Lys-Asn-Lys-Lys-Tyr-Ile-Glu-OH
SM 83.23	402 - 413	195K	Glu-Tyr-Pro-Asn-Gly-Val-Thr-Tyr-Pro-Leu-Ser-Tyr-OH
SM 83.30	595 - 606	195K	Lys-Lys-Phe-Thr-Lys-Asp-Glu-Asn-Lys-Pro-Asp-Glu-OH
SM 83.33	694 - 707	195K	Thr-Gln-Gly-Gln-Ser-Asp-Asn-Ser-Glu-Pro-Ser-Thr-Gly-Gly-OH
Random coil			
SM 83.18	277 - 287	195K	Lys-Leu-Tyr-Gln-Ala-Gln-Tyr-Asp-Leu-Ser-Thr-OH

All the peptides of 195K molecule were synthesized according to the sequence described by Holder *et al.*[5]. The N-terminal sequences of the 35K and 55K molecules were determined by sequencing 250 pm of the purified proteins using a Beckman 890 M sequencer. The residues were identified by HPLC on an ODS column using an acetonitrile/TFA gradient as described previously[11]. The 18 peptides used for the immunization experiments were synthesized using the multiple solid phase method[6] in polypropylene bags (74 μm pore size) with ~150 mg of p-methylbenzhydrylamine resin HCl (US Biochemical Corp.). The resin was deprotonated by addition of 5% diisopropylethylamine (DIEA, Aldrich) in methylene chloride (DCM) before introduction of the first amino acid. The coupling cycle was initiated by submerging bags in a solution containing equimolecular amounts of the *t*-boc amino acid (Bachem-Peninsula) and diisopropylcarbodiimide (DIPCDI-Aldrich), in a three-to-fourfold molar excess over available amine in the bag. The reaction was allowed to proceed for 60 min and the product was washed with DCM and isopropanol. The efficiency of the coupling reaction was assessed by picric acid titration. Coupling reactions were repeated if efficiencies fell below 99%. Protective groups of the newly-coupled amino acid were removed with 50% trifluoracetic acid (TFA-Pierce) in DCM. Reaction products were then washed and amino groups deprotonated with DIEA. Coupling of Asn and Gln was carried out with the addition of 1-hydroxy-benzotriazole hydrate (Aldrich) in dimethylformamide. Amino-acid side groups were protected with Bzl; Arg, His with tosyl; Cys with metoxybenzyl, Lys with Cl-Z, and Tyr with Br-z. The peptides were liberated from the resin by treatment with 2 ml of 10% anisole in anhydrous hydrogen fluoride (HF-Matheson) for 60 min at 0 °C. Twenty-four samples could be cleaved simultaneously. HF was distilled from the reaction and the product was washed 5 times with ethyl ether. The peptides were extracted in 5% acetic acid, analysed and purified by HPLC on an ODS column.

Table 3 Postchallenge parasitaemia in *Aotus* monkeys immunized with synthetic peptides

	Monkey no.	% Parasitaemia after challenge on days 4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	22	24	26	28	30	35	40	45	60	75	90
Control	358	0.1	0	0.9	0.9	3.3	7.9	10.2	28.4	Q																		
	357	0	0	0.1	0	0.5	1.6	2.8	4.0	7.4	19.0	Q																
	359	0.1	0	0.5	0.9	0.9	2.4	2.5	5.4	9.0	9.0	4.5	11.5	12.0	19.0	Q												
	239	0	0	0.8	0.5	1.5	5.0	5.6	32.5	Q																		
Mixture of SPf 35.1 SPf 55.1 and SPf 83.1	255	0	0	0.6	1.0	3.7	6.7	10.9	31.0	Q																		
	287	0	0	0.8	0.8	1.0	1.6	4.1	6.0	11.8	9.6	9.8	10.0	10.5	Q													
	251	0	0	0	0	0	0	0	0.2	0.2	1.2	3.2	ND	11.6	Q													
	275	0	0	0	0.1	0.5	0.5	0.5	2.0	4.0	6.5	6.0	6.6	ND	8.3	0.9	0.6	0.5	0.4	0.1	0	0	0	0	0	0	0	0
	288	0	0	0	0	0.2	0.1	0	1.0	0.8	2.3	ND	4.5	5.7	3.1	1.0	1.0	0.5	0.1	0	0	0	0	0	0	0	0	0
	289	0	0	0	0.2	0.1	0.2	1.0	2.3	5.4	19.4	6.8	2.7	5.4	ND	0.2	0.2	0.2	0.1	0	0	0	0	0	0	0	0	0
	286	0	0	0.1	0	0.1	0.4	0.4	3.5	3.7	3.2	ND	0.2	0.2	0	0.2	0.2	0.2	0.2	0	0	0	+					
	295	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0.3	4.4	5.5	2.1	0.2	0	0	0	0	0	0	0
	298	0	0	0	0	0	0	0	0	0	0	0	0	0	0.3	1.3	4.8	0.9	0.4	0.1	0	0	0	0	0	0	0	0
	290	0	0	0	0.1	0	0	0	0.3	0	0.7	0.1	0.7	0.4	2.5	1.1	0.6	0	0	0	0	0	0	0	0	0	0	0
	291	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+		
	297	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Postchallenge parasitaemia in *Aotus trivirgatus* monkeys immunized with synthetic peptide mixtures. Parasitaemia is expressed as the percentage of infected cells. Q, initiation of treatment with chloroquine; †, death; ND, not done. The first group of monkeys was immunized with 250 μg of each peptide, SPf 35.1 and SPf 55.1, conjugated 1:1 to BSA. The second group of monkeys was immunized with a mixutre containing equal amounts of the BSA-conjugated peptides SPf 35.1, SPf 55.1 and SPf 83.1. Controls were immunized with saline solution diluted 1:1 in Freund's adjuvant. Immunization and challenge with *P. falciparum* parasites were carried out in the same way as before. Parasitaemia was determined daily.

› patients with NEC is shown in Fig.

ieveral in vitro systems, such as pri-
ry human embryonic kidney cells, hu-
n embryonic lung fibroblasts, HEP-2,
ro, and BHK cells, did not support
growth of the viral particles. Al-
ugh attempts have been made to
ıpt the virus to a cellular substrate
t can be m re easily managed, human
ıl intestinal organ culture appears to
the only reproducible system at pres-
. Treatment f the cultures with tryp-
appeared to facilitate the infection,
ce the treated cultures gave rise to
her yields of viral particles, as seen on
ctron microscopy, than did untrypsin-
d cultures.

ın tests t date, the two strains isolat-
appear t be identical. Immunologic
ts with specific antisera should allow
ification of this finding and enable the
ablishment of possible antigenic rela-
nships with other coronaviruses. Oth-
workers have reported an association
tween coronaviruses and NEC or with
ious gastrointestinal disease in nurs-
infants. These reports were based on
servations of virus particles in stools
electron microscopy or immune elec-
ın microscopy (*19–21*). However, at-
npts t cultivate these particles were
t successful. Our results lend further
idence to the hypothesis of the exis-
ıce of a human enteric coronavirus
d suggest an association between
se virus particles and cases of NEC
served in the Dallas epidemic. Further
ıdy is needed to substantiate this asso-
ıtion, and a complete epidemiological
vestigati n of the outbreak will be re-
rted later.

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Recombinant Vaccinia Virus: Immunization Against Multiple Pathogens

Abstract. *The coding sequences for the hepatitis B virus surface antigen, the herpes simplex virus glycoprotein D, and the influenza virus hemagglutinin were inserted into a single vaccinia virus genome. Rabbits inoculated intravenously or intradermally with this polyvalent vaccinia virus recombinant produced antibodies reactive to all three authentic foreign antigens. In addition, the feasibility of multiple rounds of vaccination with recombinant vaccinia virus was demonstrated.*

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The ability to introduce endogenous inactive subgenomic fragments of vaccinia virus into infectious progeny virus by marker rescue techniques (*1*, *2*) suggested that extensions of these protocols might allow for the insertion of foreign genetic material into vaccinia virus. This was initially demonstrated by the insertion and expression of DNA sequences derived from herpes simplex virus encoding thymidine kinase (*3*, *4*). One of the notable uses of vaccinia virus expressing foreign genes is the potential generic approach for the production of live recombinant vaccines directed against heterologous pathogens. Examples of recombinant vaccinia viruses expressing the hepatitis B virus surface antigen (HBsAg) (*5*, *6*), the herpes simplex virus glycoprotein D (HSVgD) (*5*, *7*, *8*), the influenza virus hemagglutinin (InfHA) (*9*, *10*), the rabies glycoprotein (*11*, *12*), the *Plasmodium knowlesi* sporozoite antigen (*13*), and the vesicular stomatitis virus G protein (*14*) have been described. In all cases, expression of the foreign gene in vitro was, by all biochemical and biophysical criteria applied, similar to, if not identical with, the gene product synthesized under native conditions. Vaccination of laboratory animals with recombinant vaccinia produced antibodies capable of neutralizing the infectivity of correlate viruses (*5*, *7*, *9*, *12*, *14*), induced specific cytotoxic T-lymphocytes (*12*, *15*), and, significantly, protected laboratory animals on subsequent challenge with the correlate pathogen (*5*, *7*, *10*, *12*, *14*, *16*).

An advantage of vaccinia for vaccine construction is the potential for the insertion of multiple foreign genetic elements within a single vaccinia virus genome. Such a polyvalent vaccine could elicit immunity to a number of heterologous infectious diseases with a single inoculation. We report here the construction of vaccinia virus recombinants expressing multiple foreign genes. The immunological responses to inoculation obtained with these polyvalent recombinant vaccinia viruses as well as data obtained from multiple vaccinations of laboratory animals are presented.

The modification of a spontaneously occurring viable deletion mutant (*17*) of vaccinia virus to express the 1780–base pair (bp) complementary DNA (cDNA) of the RNA segment encoding the InfHA has been detailed (*9*). This recombinant virus, vP53, was used as a substrate for insertion of the HSVgD coding sequence (*18*). The recombinant vaccinia virus,

vaccines would also skew the benefit-risk ratio of vaccination to the benefit side since the benefit t be derived would be that of immunity against the total number f heterologous pathogens, whereas the risk would be that of the single vaccination.

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18. A 1.8-kb fragment derived from vaccinia Hind III M was directionally cloned into the Hind III–Sal I region of pBR322, generating pMP62. An 800-bp Sph I–Bgl II vaccinia fragment isolated from pMP62 was subjected to partial Rsa I digestion and a 200-bp Rsa I–Bgl II fragment containing an early promoter upstream from the Rsa I site was isolated. The Rsa I terminus and the Sph I site of pMP62 were modified to contain Hind III sites. The Hind III–Bgl II fragment containing an early vaccinia promoter was then ligated into the modified Sph I–Bgl II region in pMP62, generating pMP62-15 and resulting in a deletion of 0.6 kb of vaccinia sequences. The 2.5-kb Hind III–Sst I fragment containing the HSVgD (5) coding sequence was trimmed by Nru I digestion (23). The 1330-bp fragment was modified by linker addition, and the Hind III–terminated HSVgD coding sequences were inserted into the Hind III site within the vaccinia sequences of pMP62-15, generating plasmid pLP2. This plasmid was used as donor DNA for in vivo recombination (3) with vP53, and a novel recombinant vaccinia virus, vP124, containing the HSVgD sequences in addition to the InfHA sequences, was isolated by replica filter plating (3).
19. A 2.7-kb Bgl II fragment spanning the vaccinia Hind III M-K junction and containing an internal Bgl II site was inserted into the Bam HI site of pBR322H$^-$, a pBR322 derivative having a deleted Hind III site; this generated pMP18. A 360-bp Hind III–Bam HI fragment containing an early vaccinia virus promoter derived from Hind III fragment I was directionally cloned at the single Hind III–Bgl II site of pMP18, eliminating 0.1 kb of endogenous vaccinia sequences; this generated a novel plasmid pMP18PP. A Hind III–terminated HBsAg coding sequence of 1090 bp (5) was inserted at the single Hind III site of pMP18PP in proper orientation with the vaccinia promoter. This donor plasmid pPP18sAg was used as donor DNA for in vivo recombination with vP124 and a novel recombinant vaccinia virus, vP168, containing the HBsAg sequences in addition to the InfHA and HSVgD sequences was isolated by replica filter plating. Plasmid constructions as well as recombinant vaccinia viruses were confirmed by restriction analysis.
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Growth Regulation of Human Melanocytes: Mitogenic Factors in Extracts of Melanoma, Astrocytoma, and Fibroblast Cell Lines

Abstract. *Melanocytes derived from fetal or adult skin do not propagate in vitro unless cultured in the presence of factors such as 12-O-tetradecanoylphorbol 13-acetate (TPA). In a search for physiological factors regulating the growth of melanocytes, extracts of various cultured cell types were tested. Factors produced by melanoma and astrocytoma cell lines support continued proliferation of melanocytes in the absence of TPA. WI-38, a fibroblast cell line derived from human embryonic lung, was the most active source of melanocyte growth factors. No melanocyte growth-promoting activity was found in extracts of cultured neuroblastoma, renal cancer, normal keratinocytes, or renal epithelium. Nerve growth factor, epidermal growth factor, melanocyte-stimulating hormone, transforming growth factor-β, and platelet-derived growth factor did not have growth-promoting activity for melanocytes. The presence of melanocyte growth factors and TPA together resulted in the strongest mitogenic activity for melanocytes, permitting the recovery (at 20 days) of 4 to 20 times as many cells as in growth factor or TPA alone.*

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Melanocytes are the melanin pigment–producing cells of the body, which in normal human skin represent a minor cell population that undergoes mitosis only rarely (*1*). Little is known about melanocyte growth regulation, owing mainly to difficulties in obtaining sufficient numbers of melanocytes for studies in vitro. We showed earlier (*2*) that 12-*O*-tetradecanoylphorbol 13-acetate (TPA) fosters replication of melanocytes in vitro by permitting the preferential attachment of melanocytes from skin cell suspensions and stimulating them to grow. This mitogenic activity of TPA can be potentiated by cholera toxin (*2*) and isobutylmethylxanthine (*3*). In contrast to melanocytes, human melanoma cells generally grow vigorously in vitro in the absence of TPA, suggesting that their independent growth might be associated with the production of melanocyte growth factor or factors. To pursue this idea, we began a search for factors produced by melanoma and other cell types that would permit growth of melanocytes in the absence of TPA.

Melanocytes were isolated and cultured as described (*2*). To avoid fibroblast contamination, we trypsinized the cultures and purified them by immune rosetting and Percoll gradients (*4*), or we treated them with geneticin (*3*). Cultures used for experiments were free of contaminating fibroblasts as shown by leucine-aminopeptidase staining (*5*). Nonpigmented melanoma cell lines were selected for study to avoid the toxic effects of intermediate metabolites in melanin synthesis. In preliminary studies, we evaluated melanocyte growth-stimulating activity in the supernatant fluids of four melanoma cell lines. Since little or no activity was detected, we turned to cell extracts.

Figure 1 illustrates tests with extracts derived from two cell lines of neuroectodermal origin—namely, SK-MEL-131 melanoma and AO_2V_4 astrocytoma—and from the embryonic lung fibroblast line WI-38. Melanocytes cultured in the absence of TPA showed minimal [^{3}H]thymidine incorporation and no cell growth. In the presence of SK-MEL-131, AO_2V_4, or WI-38 extracts, melanocytes showed active [^{3}H]thymidine incorporation and repeated rounds of cell division. Extracts of SK-MEL-131 or AO_2V_4 resulted in a sixfold increase in [^{3}H]thymidine incorporation (as compared to melanocytes grown in the absence of added extract or TPA). Extracts of WI-38 resulted in a 20-fold increase in [^{3}H]thymidine incorporation. With regard to cell growth and division, melanocytes cultured in the absence of TPA generally round up and detach, and after 20 days cultures are lost. The effect of cell extracts on melanocyte growth parallels their effect on [^{3}H]thymidine incorporation, with extracts of WI-38 exerting the strongest mitogenic activity for melanocytes. Titration experiments showed that extracts diluted 1:500 to 1:1000 gave optimal stimulation of melanocyte growth; activity could still be detected at dilutions of 1:10,000. Growth inhibition, most likely nonspecific, was found with extracts diluted less than 1:100. Eighty percent of the growth-stimulating activity of WI-38 extracts was removed

To determine whether a primary vaccination precludes revaccination with vaccinia virus recombinants engineered to express novel foreign antigens, we used two approaches. A rabbit inoculated with a vaccinia virus recombinant expressing the HBsAg retained high titers of antibody to HBsAg for more than 1 year (Table 2). Revaccination with a vaccinia recombinant expressing the same foreign antigen led to increased levels of antibodies reactive with the HBsAg (Table 2, rabbit 488). Revaccination also induced increased antibody against vaccinia virus. Since the recombinant vaccinia harbors the genetic information encoding HBsAg, this anamnestic response is compatible with the interpretation that additional HBsAg was synthesized during replication of the recombinant vaccinia virus on revaccination. Direct evidence for the induction of antibodies reactive to a foreign antigen on revaccination of a previously immune animal was obtained as follows. A rabbit was inoculated initially with a vaccinia virus recombinant expressing the HBsAg (Table 2, rabbit 517). Antibodies to HBsAg persisted at high titer for a year beyond the initial inoculation, at which time the same rabbit was revaccinated with a vaccinia virus recombinant expressing the InfHA instead of the HBsAg. As expected, this revaccination had essentially no effect on the titer of antibodies to HBsAg. However, InfHA antibodies were detectable in the serum after the rabbit was revaccinated with the novel vaccinia virus recombinant expressing the InfHA.

Genetically engineered vaccinia virus harboring foreign genes encoding pertinent vaccine antigens can be used to immunize against a variety of foreign pathogens. Recombinant vaccinia virus can be used in a revaccination schedule either to give a booster effect or to elicit an immunological response to a different foreign antigen, even when there is already an immune response to vaccinia. In addition, one may elicit immunity against multiple foreign pathogens by constructing engineered vaccinia virus harboring coding sequences of multiple foreign antigens. Thus, a single vaccination would serve to immunize against various heterologous pathogens. The ability of the virus to accept large quantities of foreign DNA makes the construction of polyvalent recombinant vaccinia viruses possible. Indeed, as much as 20 (9) to 25 kb (22) of foreign DNA can be stably inserted into vaccinia virus vectors. Additional space for the insertion of foreign DNA can be obtained by using defined viable deletion mutants of the virus. One can readily think of polyvalent vaccinia virus vaccines genetically engineered to express foreign genetic information encoding several dozen antigens. Whether the immunological system could respond adequately to such an intrusion remains to be established. Nevertheless, as we have shown in this report, animals respond to at least three novel foreign antigens even in the presence of the several hundred endogenous vaccinia antigens that are produced on vaccinia infection. Polyvalent vaccinia-vectored vaccines would be useful in circumstances in which multiple serotypes of a given pathogen need to be considered, for example, in the construction of a veterinary vaccine directed against foot-and-mouth disease. The use of polyvalent recombinant vaccinia virus

Table 1. Immunological response to vaccination with a recombinant vaccinia expressing multiple foreign genes. Rabbits were inoculated with 5×10^7 plaque-forming units in 0.5 ml either intravenously (rabbit 257) or intradermally (rabbit 288) with a vaccinia recombinant expressing the HBsAg, HSVgD, and InfHA genes, and the immunological response was followed. Sera were tested for antibodies to HBsAg with the commercially available AUSAB radioimmunoassay (RIA) kit (Abbott); titers are expressed in RIA units per milliliter of serum as defined by the manufacturer. Antibodies to InfHA were measured by hemagglutination inhibition tests performed with chicken erythrocytes and four hemagglutinin units. The reciprocal of serum dilution is indicated. Plaque-reduction assays monitoring reduction of HSV or vaccinia virus infectivity were performed on CV-1 cells. The reciprocal of serum dilution giving greater than 50 percent reduction in plaque number is shown.

Weeks after inoculation	Anti-HBsAg (RIA units)	Anti-InfHA	Anti-HSV	Anti-vaccinia
		Rabbit 257		
1	160	160	640	6,400
2	160	160	5,120	6,400
3	160	320	2,560	12,800
4	670	320	2,560	12,800
5	1,840	640	1,280	12,800
		Rabbit 288		
1	80	20	<40	<40
2	160	80	320	400
3	360	40	160	400
4	760	80	320	400
5	760	80	160	400

Table 2. Revaccination of rabbits with recombinant vaccinia virus. Rabbits inoculated intradermally with 1.8×10^7 plaque-forming units of a vaccinia virus recombinant expressing the HBsAg were revaccinated at 56 weeks with an equivalent dose of a vaccinia virus recombinant expressing HBsAg (rabbit 488) or revaccinated at 54 weeks with a novel vaccinia virus recombinant expressing the InfHA (rabbit 517). Anti-HBsAg antibody levels were determined using a commercially available RIA kit from Abbott Laboratories and are noted in RIA units ($\times 10^{-3}$) per milliliter of serum as defined by the manufacturer. For plaque-reduction assays, vaccinia virus was mixed with dilutions of antisera and kept at 4°C overnight until they were titrated on CV-1 monolayers. The percent reduction of plaques obtained on CV-1 monolayers are indicated as the reciprocal of the serum dilution. Antibody to InfHA was determined by standard hemagglutination inhibition tests with chicken erythrocytes and four HA units. Reciprocal of serum dilution is shown.

Weeks after inoculation	Anti-HBsAg (RIA units)	Vaccinia virus plaque reduction (%)		Anti-InfHA
		1600	6400	
		Rabbit 488		
1	0.1	63	12	
3	0.4	69	35	
6	18.4	82	12	
54	6.8	41	0	
57	32.7	85	4	
62	24.3	90	67	
		Rabbit 517		
3	0.2			<10
6	16.0			<10
9	72.0			<10
40	128.0			<10
54	72.0			<10
55	72.0			40
56	63.0			160
57	43.0			80
58	63.0			160
59	63.0			320
60	63.0			640

vP124, containing both the InfHA and the HSVgD was used in turn as substrate for insertion of the HBsAg coding sequence, thus generating the triple vaccinia virus recombinant vP168 (*19*). All three foreign coding sequences were inserted at nonessential sites in the vaccinia genome, and transcription of the inserted genes was right to left relative to the vaccinia genome. The HSVgD, the HBsAg, and the InfHA sequences were inserted 23.9, 25.1, and 34.1 kilobases (kb), respectively, from the left terminus of the vaccinia genome, as referenced, to the L variant vaccinia genotype (*17*) (Fig. 1A).

The recombinant vaccinia virus, vP168, was plaque-purified, amplified to high titer, and highly purified on sucrose gradients (*20*). The DNA was extracted and cleaved with Hind III or Bam HI restriction endonucleases. The DNA fragments were separated on agarose, blotted onto GeneScreen Plus (DuPont) by the Southern method (*21*), and probed with ^{32}P-labeled nick-translated sequences homologous to the foreign genes (Fig. 1B). Hind III digestion released a 1090-bp fragment (Fig. 1B, lan 2) equivalent in size to a purified HBsAg sequence (lane 1) and a 1338-bp fragment (lane 2) equivalent in size to a purified HSVgD sequence (lane 3). The third band in lane 2 represents the internal vaccinia Hind III F fragment harboring the InfHA sequences inserted at the unique Bam HI site (*9*) (Fig. 1A). The InfHA sequences, which were excised from the recombinant vaccinia genome by Bam HI digestion (lane 4), migrated to a size of 1780 bp, equivalent to the size of purified HA sequen e (Fig. 1B, lane 5). With Bam HI stion the HSVgD sequences reside w... in a larger vaccinia fragment of approximately 4 kb. HBsAg sequences are contained in the same 4-kb fragment as well as in a larger 9-kb fragment (Fig. 1A). The results with a mixed HSVgD, HBsAg, and InfHA probe (Fig. 1B, lanes 1 to 5) were confirmed by the use of individual HBsAg, HSVgD, and InfHA probes (Fig. 1B, lanes 6 to 8, respectively). The presence of additional faint bands containing HSVgD sequences (Fig. 1B, lanes 4 and 7) are due to minor rearrangements of the vaccinia genome.

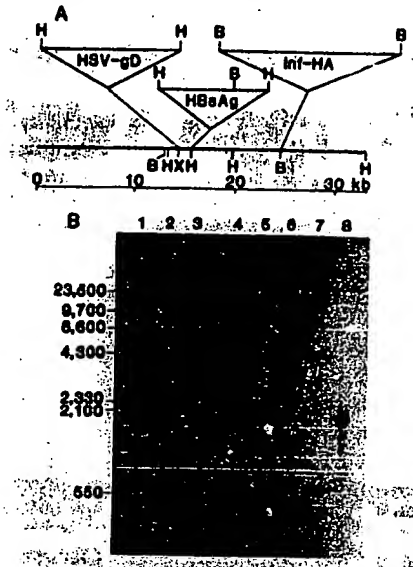

Fig. 1. (A) Structure of the vaccinia virus recombinant harboring the HSVgD, HBsAg, and InfHA coding sequences. The leftmost 30 kb of the vaccinia S variant genome (*17*) encompassing the terminal Hind III F, M, K, and internal F fragments are shown. The restriction sites identified in Fig. 1A are B (Bam HI) and H (Hind III). The X denotes the deletion of approximately 0.6 kb of vaccinia sequences described in the construction of pMP62-15 (*18*). (B) Southern blot hybridization analysis of the vaccinia virus recombinant vP168. DNA was extracted from purified vaccinia virus, digested with restriction endonucleases, and blotted to GeneScreen Plus after electrophoretic separation of the DNA fragments on agarose. vP168 DNA was digested with Hind III (lane 2) or with Bam HI (lane 4 and lanes 6 to 8) and probed with a mixture (lanes 1 to 5) or single (lanes 6 to 8) ^{32}P-labeled nick-translated HBsAg, HSVgD, and InfHA sequences. Purified DNA fragments containing the coding sequences for HBsAg (lane 1), HSVgD (lane 3), and InfHA (lane 5) were run as standards. Sizes of the fragments were obtained with Hind III–cleaved λ DNA as markers.

The expression in vitro of the InfHA, HSVgD, and HBsAg genes by the vaccinia virus recombinant vP168 was detected by means of serological tests (Fig. 2). BSC-40 cells infected with vP53, containing the InfHA and its engineered offspring vP124 and vP168, were shown to express the InfHA by radioimmunoassay. No radioimmunological reactivity with antiserum to InfHA was observed in the cells infected with wild-type (thymidine-kinase–deficient) vaccinia virus, VTK$^-$79. Similar radioimmunoassays with antiserum to herpes simplex virus gave positive signals of HSVgD expression when BSC-40 cells were infected with the recombinant vP124 and it. engineered offspring vP168. No serological reactivity was observed when the cells were infected with either VTK$^-$79 or vP53. When a commercially available radioimmunoassay was used to detect HBsAg expression, positive results were obtained with the vP168 recombinant, whereas no serological reactivity was demonstrated with cells infected with its progenitors vP124 and vP53 or with VTK$^-$79.

Rabbits were inoculated either intravenously or intradermally with the vaccinia virus recombinant vP168. Serum samples were obtained weekly and screened for antibodies t the foreign antigens (Table 1). Both rabbits responded by making antibodies to all three of the foreign antigens synthesized under vaccini virus regulation. The intravenous route of inoculati n also provides a criterion for the absence of toxicity in the preparation of the vaccinia recombinant.

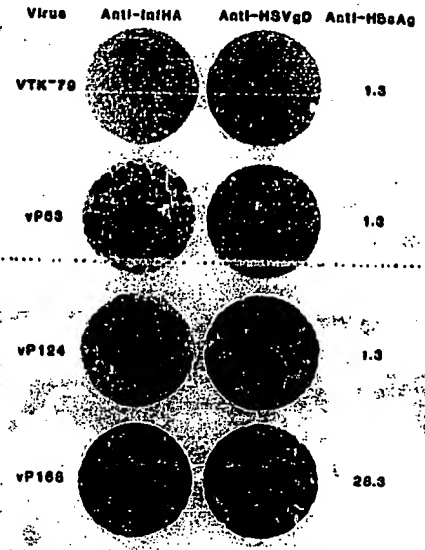

Fig. 2. Detection of InfHA, HSVgD, and HBsAg expression by vaccinia virus recombinants. BSC-40 cells were infected with either wild-type vaccinia virus, VTK$^-$79, or the vaccinia virus recombinants containing coding sequences for the InfHA (vP53), the InfHA and HSVgD (vP124), or the InfHA, HSVgD, and HBsAg (vP168). Two days after infection, the plaques were visualized by neutral red staining. The monolayers were exposed to antiserum to InfHA or to HSV at room temperature for 1 hour. Unreacted antibody was removed by washing in phosphate-buffered saline, and ^{125}I-labeled protein A from *Staphylococcus aureus* was added as previously described (*9*). Unreacted ^{125}I-labeled protein A was removed by washing in saline. The monolayers were imprinted on nitrocellulose filters, dried, and radioautographed. Film densities indicate presence of antibody-bound ^{125}I-labeled protein A. Unlike the InfHA and the HSVgD, which are localized on the infected cell membrane, the HBsAg synthesized by vaccinia virus is excreted from the infected cells (*5*). Therefore, BSC-40–infected cell extracts were prepared by three cycles of freezing and thawing, and the presence of HBsAg was detected with a commercially available radioimmunoassay kit AUSRIA II (Abbott). The numbers represent the positive-to-negative ratios (P/N) as defined by the manufacturer. The horizontal columns represent BSC-40 cells infected with the wild-type VTK$^-$79 or the vP53, vP124, or vP168 recombinant vaccinia viruses. The vertical columns represent radioimmunoassays of unfixed, infected BSC-40 monolayers in which antiserum to InfHA or to HSVgD was used, or the P/N ratio as detected with the AUSRIA II kit.

SPf 55.1 SPf 35.1 and SPf 83.1, using the same immunization schedule as before. Monkeys in this experimental group devel ped a higher antibody titre compared to the previous group (Taole 1).

F ur of the eight *Aotus* monkeys immunized with two peptides (55.1 and 35.1) developed the disease similarly to the controls; the remaining four developed parasitaemias lower than 10% and spontaneously recovered. In these animals parasitaemias had not developed by day 180, in contrast to animals immunized with individual peptides.

Three of the six monkeys immunized with the three-peptide mixture (83.1, 55.1 and 35.1) developed very mild infection with parasitaemia maxima of 5%, peaking 10–15 days later than in the control group, followed by spontaneous recovery. The remaining three monkeys of this same group showed no signs of disease and no parasites at all were detected in blood smear samples (Table 3) up to 180 days (shown until day 90) after challenge. There seems to be no direct correlation between antibody titre and protection and, contrary to what has been suggested[8,9], there was no correlation of protection with predicted peptide structure. This could be due to the limitations of the method we used to predict these structures.

Recently, Cheung *et al.*[13] also obtained partial sterilizing protection in a *P. falciparum* vaccine trial in *Saimiri* monkeys with a 31-residue peptide that contains our SPf 83.1 peptide sequence. In contrast to our results, Collins *et al.*[14] described partial sterilizing protection in some *Aotus* monkeys immunized with a genetically engineered fragment of RESA corresponding t a polymeric form of our non-protective SPf 155.1 synthetic monomer. This difference could be due to the fact that we were working with the monomeric form of the repeated octapeptide. The three-peptide mixture may have provided better protection due to the fact that it contains fragments from different blood-stage antigens. One should remember, however, that because of the limited number of monkeys tested with this three-peptide mixture, the possibility remains that the protection offered could be due to a combination of two peptides.

These results show that the combination of the three peptides SPf 35.1 (Tyr-Gly-Gly-Pro-Ala-Asn-Lys-Lys-Asn-Ala-Gly), SPf 55.1 (Asp-Glu-Leu-Glu-Ala-Glu-Thr-Gln-Asn-Val-Tyr-Ala-Ala) and SPf 83.1 (Tyr-Ser-Leu-Phe-Gln-Lys-Glu-Lys-Met-Val-Leu), synthesized according to the amino acid sequences of polypeptides which had already been shown to offer complete r partial immunity against experimental infection, are capable f inducing partial sterilizing or complete immunity in the vaccinated animals. This combination of synthetic peptides is worth considering as a potential vaccine against malaria.

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Induction f c-*fos*-like proteir in spinal cord neurons f ll wing sensory stimulati n

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It has been suggested that the proto-oncogenes c-*fos* and c-*myc* participate in the control of genetic events which lead to the establishment of prolonged functional changes in neurons[1,2]. Expression of c-*fos* and c-*myc* are among the earliest genetic events induced in cultured fibroblast and phaeochromocytoma cell lines by various stimuli including growth factors, peptides and the intracellular second messengers diacylglycerol, cAMP and Ca^{2+} (refs 3–11). We report here that physiological stimulation of rat primary sensory neurons causes the expression of c-*fos*-protein-like immunoreactivity in nuclei of postsynaptic neurons of the dorsal horn of the spinal cord. Activation of small-diameter cutaneous sensory afferents by noxious heat or chemical stimuli results in the rapid appearance of c-*fos*-protein-like immunoreactivity in the superficial layers of the dorsal horn. However, activation of low-threshold cutaneous afferents results in fewer labelled cells with a different laminar distribution. No c-*fos* induction was seen in the dorsal root ganglia, gracile nucleus or ventral horn. Thus, synaptic transmission may induce rapid changes in gene expression in certain postsynaptic neurons.

Somatosensory information enters the central nervous system (CNS) via primary afferent neurons which synapse on to cells in the dorsal horn of the spinal cord. Activation of small-diameter afferents can lead to the development of long-lasting changes in both the threshold of spinal reflexes[12,13] and the size of the receptive fields of dorsal horn neurons[14]. Because of the suggested link between the expression of the c-*fos* proto-oncogene and the induction of persisting changes in excitable cells[1,2] we sought evidence that c-*fos* protein is expressed in spinal cord neurons after sensory stimulation.

The following stimuli were applied to activate primary afferent inputs. First, we used cutaneous application and intramuscular injection of the irritant mustard oil (5% in paraffin oil), which produces neurogenic plasma extravasation and stimulation of unmyelinated C polymodal and Aδ myelinated nociceptive afferents[13,15]. Second, we applied radiant heat (ten localized pulses at 40 °C or 52 °C for 20 s at 90-s intervals) which drives C polymodal nociceptors, although a minor contribution—from heat sensitive Aδ high threshold mechanoreceptors cannot be excluded[16]. Third, we used non-noxious brushing of hairs and gentle manipulation of joints (continuous for 15 min) which activates myelinated Aβ and unmyelinated C low-threshold mechanoreceptors, Aβ/δ hairs and Aα proprioceptive afferents[16–19].

Two hours after cutaneous application of mustard oil to the hind limb ($n = 4$), p55 $^{c\text{-}fos}$-like immunoreactivity was localized to nuclei in a restricted region of the ipsilateral medial dorsal horn of lumbar segments 3 and 4, as is appropriate for this cutaneous input (Fig. 2*b*). By light microscopy, counterstained cells possessed large nuclei (10 μm maximum diameter) and, at least in deep layers IV and V, were clearly neuronal (Fig. 2*e*). In m re superficial layers cells could not b classified unequivocally as neuronal because of their small size. H wever, by electron microscopy, all f a sample f 25 superficial cells had dendritic processes and a cytoplasmic and nuclear m rph l gy characteristic of neurons[20] (data n t sh wn). Most labelled nuclei were f und in layers I (marginal) and II (substantia

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(57) Abstract

A recombinant protein is provided which comprises peptides derived from different stages in the life cycle of the parasite *Plasmodium falciparum*. The protein is useful as a reagent and, when combined with a pharmaceutically–acceptable vehicle or carrier, is useful as a vaccine against the malarial parasite *Plasmodium falciparum*. A genetic construct used to produce this recombinant protein vaccine is also described. In addition, antibodies to this recombinant protein are provided which are useful for the detection and measurement of peptides derived from different stages in the life cycle of the parasite *Plasmodium falciparum*.

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RECOMBINANT MULTIVALENT MALARIAL VACCINE AGAINST *PLASMODIUM FALCIPARUM*

This invention was made by the Centers for Disease Control and Prevention, an agency of the United States Government. Therefore the United States Government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates generally to the development and use of a gene encoding a recombinant protein useful as a multivalent and multistage malaria vaccine and more specifically relates to a recombinant antigenic protein useful for preventing or treating *P. falciparum* malarial infections.

BACKGROUND OF THE INVENTION

Malaria is a parasitic infection known to be produced by the *Plasmodium* species *P. falciparum, P. vivax, P. ovale,* and *P. malariae.* Humans become infected following the bite of an infected anopheline mosquito, the host of the malarial parasite. Malaria occasionally occurs in humans following a blood transfusion or subsequent to needle-sharing practices as used by drug addicts.

When an infected anopheline mosquito bites an individual, sporozoites present in the mosquito's saliva are injected into the blood. The initial development of parasites occurs in the liver and is referred to as the liver stage, or the hepatic or exoerythrocytic phase. In this phase, the sporozoite grows and divides, producing numerous tissue merozoites. These merozoites rupture the hepatocyte and enter the circulation. Some merozoites attach to receptor sites on red blood cells, penetrate the plasmalemma and begin a development phase known as the asexual, erythrocytic cycle. Within the erythrocyte, the parasite is recognizable as a ring-stage trophozoite. These trophozoites enlarge, divide and attain the schizont stage. After successive nuclear divisions, the erythrocyte ruptures, releasing merozoites which attach to receptors on erythrocytes and thus begin another

erythrocytic cycle. In *P. vivax* and *P. ovale,* hepatic parasites persist and may lead to a relapse of the disease months or years after the initial infection.

Some merozoites that enter red blood cells develop into male and female gametocytes. When a mosquito bites an individual possessing erythrocytic gametocytes and ingests them, the gametocytes are fertilized in the stomach of the mosquito and mature into sporozoites that migrate to the salivary glands. In this manner, the mosquito is capable of biting and infecting another individual.

Malaria is one of the most common infections of humans. It is estimated that malaria parasites cause about 300-500 million illnesses and 3 million deaths each year. Most of the severe morbidity and mortality occurs in children and pregnant women, and is caused by *P. falciparum* (World Health Organization (1989) *Weekly Epidemiol. Res.* 32, 241-247). While sub-Saharan Africa accounts for more that 90% of these cases, malaria is a serious public health problem for nonimmune individuals and servicemen and servicewomen traveling through and/or stationed in malarious regions of the world. Clinical manifestations of malarial infection which may occur include blackwater fever, cerebral malaria, respiratory failure, hepatic necrosis, and occlusion of myocardial capillaries. An effective vaccine that prevents or reduces infection and minimizes morbidity and mortality will be a very useful tool for the control and prevention of this disease.

The development of an effective malaria vaccine represents one of the most promising approaches for providing cost-effective intervention along with other control measures currently available. Over the last decade there has been considerable progress in the understanding of immune mechanisms involved in protection against parasites and clinical illness. Several malarial antigens have been identified for their ability to confer protection against malaria.

Three main types of malarial vaccines are currently under research and development, based on stages of the parasite's life cycle. The three vaccines are generally directed to the following stages in the life cycle: 1) blood stage, including the asexual blood stage; 2) the sexual stages; and 3) preerythrocytic stages, including the liver stage. Antigens from each of these stages have been identified, the most promising being antigens from the following proteins: circumsporozoite protein (CSP) and SSP-2 protein of the sporozoite stage; the antigen (LSA-1) of the liver stage; the merozoite surface protein-1 (MSP-1), merozoite surface protein-2 (MSP-2), the rhoptry associated protein-1 and -2 (RAP-1 and RAP-2), the erythrocyte binding antigen-175 (EBA-175) and apical membrane antigen-1 (AMA-1) of the asexual blood stage; and the ookinete antigen Pfs 25 and the gamete specific antigen Pfg27 of the sexual stage.

Therefore, what is needed is a single vaccine that provides immunogenicity or confers immunity against various stages in the life cycle of the malarial parasite,

particularly *P. falciparum*, to treat, minimize or prevent infection and reduce associated morbidity and mortality.

SUMMARY OF THE INVENTION

An antigenic recombinant protein, method of making the protein, genetic construct encoding the protein, antibodies to the protein, pharmaceutical composition containing the protein, and a method for the treatment, prevention or reduction of malarial infection by administering the protein to a human or animal are provided. The protein and anti-protein antibodies are useful as research or diagnostic reagents for the detection of the *Plasmodium* species *P. falciparum* in a biological sample. When administered to human or nonhuman animals, the protein is effective against malaria by conferring immunogenicity or immunity against various stages in the life cycle of the malarial parasite *P. falciparum.*

The antigenic recombinant protein is prepared by constructing a gene that encodes stage-specific antigenic determinants. The gene is added to a vector and is then expressed in a suitable expression system, such as a baculovirus system, to produce a single protein that confers immunity against different stages in the malarial life cycle of *P. falciparum,* or provides immunogenicity against epitopes from different stages in the life cycle of the parasite. In the present invention, these stages are the sporozoite stage, the liver stage, the blood stage and the sexual stage (also known as the gametocyte stage). By using a combination of antigens or epitopes derived from different stages in the life cycle of a malarial parasite, the protein constitutes an efficacious, cost-effective, and sustainable multicomponent vaccine for use in malaria control programs. The protein, in a pharmaceutically acceptable carrier, specifically provides a multivalent and multistage vaccine for malaria caused by the parasite *P. falciparum.*

The immunogenic regions of the various stage-specific antigens of *P. falciparum* used to construct the gene encoding the antigenic recombinant protein are selected based on immune response studies in clinically immune adults and *in vitro* immune response studies using peptides and/or antibody reagents. The resulting synthetic gene is sequence-confirmed and expressed in a baculovirus expression system. The preferred antigenic fragments used to make the coding sequences used in construction of the gene are shown in Table 1. The nucleotide sequence of the preferred gene is shown in SEQ ID NO:1. The amino acid sequence of the preferred recombinant protein encoded by the gene, referred to herein as CDC/NIIMALVAC-1, is shown in SEQ ID NO. 2. The recombinant protein in a pharmaceutically acceptable carrier is useful as a multivalent, multistage vaccine for *P. falciparum* malaria.

The vaccine described herein is a cost-effective, health-promoting intervention for controlling, preventing or treating the incidence of malaria. The vaccine is useful for reducing sickness, morbidity, mortality and the cost of medical care throughout the world.

Similarly, the vaccine is useful for preventing or reducing malarial infection in U.S. citizens and military personnel traveling or living in regions of the world where malaria is present. The vaccine is also useful for decreasing the severity of the malarial disease process when administered after initial infection with *P. falciparum.*

The vaccine is immunogenic as confirmed by its ability to elicit immune responses against both the vaccine protein and the *P. falciparum* parasite. *In vitro* tests of protection conferred by the vaccine against blood stage malarial parasites reveal that antibodies against this vaccine inhibit reproductive growth of *P. falciparum.* The vaccine also induces multiple layers of immunity to different stages in the parasitic life cycle of *P. falciparum.*

It is therefore an object of the present invention to provide a multivalent, multistage vaccine against malaria.

Another object of the present invention is to provide a multivalent, multistage vaccine against malaria caused by *P. falciparum.*

Yet another object of the present invention is to provide a vaccine against malaria that is effective in inhibiting reproductive growth of the parasite within a human or animal after initial infection.

Still another object of the present invention is to provide a gene useful as a DNA vaccine, or for production of a recombinant protein in various expression systems, the recombinant protein containing antigenic epitopes to various stages of a malarial *Plasmodium* species, particularly *P. falciparum.*

Another object of the present invention is to provide a vector comprising a gene useful for production of a recombinant protein in various expression systems, the protein containing antigenic epitopes to various stages of a malarial *Plasmodium* species, particularly *P. falciparum.* This vector may be used for a variety of purposes including but not limited to administration to animals and humans, and for transfection of cells.

Yet another object of the present invention is to provide a recombinant protein containing antigenic epitopes to various stages of *P. falciparum* that may be used as a reagent or a multivalent, multistage antimalarial vaccine.

It is another object of the present invention to provide a method for conferring immunity against different stages in the life cycle of the malarial parasite, *P. falciparum.*

Another object of the present invention is to provide a method of vaccination against malaria caused by infection with *P. falciparum.*

It is another object of the present invention to provide a method to reduce morbidity and mortality associated with malarial infection by preventing malarial infection and also ameliorating the morbidity and mortality associated with malaria after initial infection with the parasite, *P. falciparum.*

Another object of the present invention is to provide antibodies against a recombinant protein containing antigenic epitopes to various stages of *P. falciparum*, that are useful as research or diagnostic reagents for the detection and measurement of *P. falciparum* in a biological sample.

Yet another object of the present invention is to provide a more effective, simpler and economical vaccine for conferring immunogenicity to different stages in the life cycle of *P. falciparum* than prior art vaccines.

An advantage of the anti-malaria vaccine of the present invention is that it confers immunogenicity against several stages or all stages in the life cycle of *P. falciparum* with administration of a single vaccine, as opposed to multiple injections for each stage of the life cycle of the parasite.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic map of the synthetic gene encoding production of the recombinant protein CDC/NIIMALVAC-1. Locations of epitopes in CDC/NIIMALVAC-1 are indicated by the codes which correspond to codes in Table 1.

Figure 2 is a flow chart revealing the strategy employed in the synthesis of the gene encoding production of the recombinant protein CDC/NIIMALVAC-1.

Figure 3 is a graph showing antibody responses in sera as measured by ELISA. Rabbits were immunized with purified CDC/NIIMALVAC-1 using different adjuvant formulations at weeks 0, 3, 6, 9 as indicated by arrows. Antibody responses to CDC/NIIMALVAC-1 in the sera from rabbits receiving Freund's adjuvant (open diamond), copolymer (solid square), and aluminum hydroxide (open circle) as adjuvant were measured. Titers were determined based on the highest dilution of the samples that generated an optical density (OD) greater than the cutoff value (mean plus three standard deviations of pre-immunization sera). ODs lower than the cutoff value at 1:50 dilution were considered negative responses.

Figure 4 is a graph showing binding of IgGs. Purified antibodies at concentration of 50 μg/ml were presented at 5 μl/min to a BIAcore sensor cell loaded with CDC/NIIMALVAC-1 protein. A chart on top left represents initial velocities of association (450-500 sec).

Figure 5 is a graph showing the antibody response (antibody titer, log scale) to CDC/NIIMALVAC-1 in inbred mice of different genetic backgrounds as a function of time in days after the first immunization. Shown are C57 mice receiving alum (solid diamond) or copolymer (open square), B10.BR mice receiving alum (solid triangle) or copolymer

(open circle), B10.D2 mice receiving alum (solid square) or copolymer (closed circle). All mice received a booster injection at 14 days.

DETAILED DESCRIPTION OF THE INVENTION

An antigenic recombinant protein containing immunogenic malarial epitopes from different stages of the malarial parasite life cycle; a method of making the protein, including a genetic construct from which the protein is produced; antibodies to the protein; a pharmaceutical composition containing the protein, useful as a malarial vaccine; and a method for treating, preventing or reducing malarial infection by administering the composition to a human or animal are described herein. The genetic construct includes coding sequences for different peptide fragments obtained from different stages in the life cycle of a malarial parasite, preferably *P. falciparum*. The genetic construct also includes epitopes chosen to enhance recognition by cells of the immune system of the protein expressed from the genetic construct. A preferred genetic construct includes coding sequences for a signal peptide, for a polyhistidine sequence useful for purification of the protein, a universal T-helper epitope, and at least one epitope from each stage in the life cycle of *P. falciparum*. The preferred genetic construct has the nucleotide sequence of SEQ ID NO:1, or a nucleotide sequence having conservative nucleotide substitutions, as defined in the definitions, that do not significantly alter the function of the expressed recombinant protein in an adverse manner.

The genetic construct is expressed in an expression system, such as a baculovirus expression system, to produce a recombinant protein. The preferred protein is the protein referred to herein as CDC/NIIMALVAC-1, which has the amino acid sequence set forth in SEQ ID NO:2, or an amino acid sequence having amino acid substitutions as defined in the definitions that do not significantly alter the function of the recombinant protein in an adverse manner. The protein is combined with a pharmaceutical carrier and is used as a multivalent vaccine to confer immunity to the different stages in the life cycle of the malarial parasite, *P. falciparum*, when combined with a pharmaceutically acceptable carrier and administered in an effective amount to a human or animal. The present invention specifically provides a multivalent and multistage vaccine useful for preventing and treating malaria caused by *P. falciparum*. The present invention also provides polyclonal and monoclonal anti-protein antibodies produced after immunization with the recombinant protein which are useful, as is the protein, as research or diagnostic reagents in an assay for the detection or monitoring of malarial infection, particularly to detect malarial infection caused by *P. falciparum*. The antibodies are also useful for inducing passive immunization. Some of these results have been published by Shi *et al., Proc. Natl. Acad. Sci. USA* 96:1615-1620, the entirety of which is herein incorporated by reference.

Definitions

The terms "a", "an" and "the" as used herein are defined to mean one or more and include the plural unless the context is inappropriate.

The term "multivalent" as used herein is defined to mean more than one epitope.

The term "multistage" as used herein is defined to mean more than one stage in the life cycle of *P. falciparum*. These stages include the sporozoite stage, the liver stage, the blood stage and the sexual stage.

"Peptides", "polypeptides" and "oligopeptides" are chains of amino acids (typically L-amino acids) whose alpha carbons are linked through peptide bonds formed by a condensation reaction between the carboxyl group of the alpha carbon of one amino acid and the amino group of the alpha carbon of another amino acid. The terminal amino acid at one end of the chain (*i.e.*, the amino terminal) has a free amino group, while the terminal amino acid at the other end of the chain (*i.e.*, the carboxy terminal) has a free carboxyl group. As such, the term "amino terminus" (abbreviated N-terminus) refers to the free alpha-amino group on the amino acid at the amino terminal of the peptide, or to the alpha-amino group (imino group when participating in a peptide bond) of an amino acid at any other location within the peptide. Similarly, the term "carboxy terminus" (abbreviated C-terminus) refers to the free carboxyl group on the amino acid at the carboxy terminus of a peptide, or to the carboxyl group of an amino acid at any other location within the peptide.

Typically, the amino acids making up a peptide are numbered in order, starting at the amino terminal and increasing in the direction toward the carboxy terminal of the peptide. Thus, when one amino acid is said to "follow" another, that amino acid is positioned closer to the carboxy terminal of the peptide than the preceding amino acid.

The term "residue" is used herein to refer to an amino acid (D or L) or an amino acid mimetic that is incorporated into a peptide by an amide bond. As such, the amino acid may be a naturally occurring amino acid or, unless otherwise limited, may encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (*i.e.*, amino acid mimetics). Moreover, an amide bond mimetic includes peptide backbone modifications well known to those skilled in the art.

Furthermore, one of skill in the art will recognize that individual substitutions, deletions or additions in the amino acid sequence of the protein, or in the nucleotide sequence encoding for the amino acids in the protein, which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are conservatively modified variations, wherein the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Antigenic Peptide Production

When the antigenic epitope peptides are relatively short in length (*i.e.*, less than about 50 amino acids), they are often synthesized using standard chemical peptide synthesis techniques. Solid phase synthesis, in which the C-terminal amino acid of the sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids in the sequence, is a preferred method for the chemical synthesis of the antigenic epitopes described herein. Techniques for solid phase synthesis are known to those skilled in the art.

Alternatively, the antigenic epitopes described herein are synthesized using recombinant nucleic acid methodology. Generally, this involves creating a nucleic acid sequence that encodes the peptide or polypeptide, placing the nucleic acid in an expression cassette under the control of a particular promoter, expressing the peptide or polypeptide in a host, isolating the expressed peptide or polypeptide and, if required, renaturing the peptide or polypeptide. Techniques sufficient to guide one of skill through such procedures are found in the literature.

While the antigenic epitopes are often joined directly together, one of skill will appreciate that the antigenic epitopes may be separated by a spacer molecule such as, for example, a peptide, consisting of one or more amino acids. Generally, the spacer will have no specific biological activity other than to join the antigenic epitopes together, or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of the spacer may be selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity.

Once expressed, recombinant peptides, polypeptides and proteins can be purified according to standard procedures known to one of skill in the art, including ammonium sulfate precipitation, affinity purification through columns or other methods commonly known, column chromatography, gel electrophoresis and the like. Substantially pure compositions of about 50 to 95% homogeneity are preferred, and 80 to 95% or greater homogeneity are most preferred for use as therapeutic agents.

One of skill in the art will recognize that after chemical synthesis, biological expression or purification, the antigenic peptide epitopes, polypeptides and proteins may possess a conformation substantially different than the native conformations of the

constituent peptides. In this case, it is often necessary to denature and reduce the polypeptide and then to cause the polypeptide to refold into the preferred conformation. Methods of reducing and denaturing proteins and inducing refolding are well known to those of skill in the art.

Recombinant Protein Production

The method of producing the recombinant protein CDC/NIIMALVAC-1 involves the following steps: 1) selecting antigenic components, preferably antigenic peptides, from different stages in the life cycle of *P. falciparum,* that are involved in conferring immunologic protection; 2) optionally selecting a signal peptide sequence, such as melittin, optionally selecting other protein or peptide epitopes useful as T-cell helpers such as tetanus toxoid, and optionally selecting protein or peptide epitopes from *P. falciparum* involved in T-cell and B-cell recognition; 3) generating gene fragments comprised of nucleotide sequences that are complementary to the selected protein fragments; 4) assembling the gene fragments to create a gene, preferably a gene having the nucleotide sequence of SEQ ID NO:1, that encodes a novel recombinant protein, preferably the protein referred to herein as CDC/NIIMALVAC-1 having the amino acid sequence of SEQ ID NO:2; 5) cloning the gene into an expression vector so that it may be expressed in an expression system; and 6) expressing the recombinant protein in the expression system. The expressed recombinant protein is then recovered and purified. This protein is combined with a pharmaceutically acceptable vehicle or carrier and is administered as a multivalent, antimalarial vaccine to humans and nonhuman animals. The vaccine is administered in an amount effective to confer immunity against infection caused by *P. falciparum*, and particularly, to confer immunogenicity or immunity against different stages in the life cycle of *P. falciparum*.

Compared to vaccines directed to a single stage in the life cycle of malaria, the multivalent and multistage *P. falciparum* vaccine of the present invention induces multiple "layers" of immunity which significantly increase the chances of neutralizing all stages in the life cycle of the malaria parasite. The method of the present invention permits synthesis of a gene that contains coding sequences for several protective/immunodominant malarial epitopes of the malarial parasite *P. falciparum*.

A potential concern with the design of a synthetic gene encoding multiple epitopes is that the tandem arrangement of epitopes in the recombinant protein may induce antigenic competition, thus rendering immunizations ineffective in inducing immune responses. As shown in the examples and figures herein, the results from an immunization study involving the administration of CDC/NIIMALVAC-1 to mice and rabbits alleviate this concern. CDC/NIIMALVAC-1 is immunogenic. The recombinant protein antigen is recognized by antibodies directed against the B-cell epitopes of the construct. The vaccine

is also antigenic since the immunization of rodents and rabbits induced antibodies that react with the protein vaccine, and also with the sporozoite and infected red blood cells. In addition, results of the experiments performed to evaluate the protective effects of immunization with CDC/NIIMALVAC-1 show that murine and rabbit antibodies against CDC/NIIMALVAC-1 inhibit parasite growth, as determined by the growth inhibition assay (GIA) and the antibody-dependent cellular inhibition (ADCI) assay.

The data set forth in the examples demonstrate that the protein, CDC/NIIMALVAC-1, in the multicomponent *P. falciparum* vaccine, induces "multiple layers" of immunity, and that anti-CDC/NIIMALVAC-1 antibodies recognize different stages of the life cycle of the malarial parasite.

Construction of the Recombinant Gene

Immunogenic regions of various stage-specific antigens are identified by immune response studies in clinically immune adults and immune response studies performed *in vitro* using peptides and antibody reagents. Short, single-stranded DNA fragments complementary to the different epitopes are synthesized by methods known to those skilled in the art. Different DNA fragments are annealed to create a synthetic multicomponent gene by a three step polymerase chain reaction (PCR) amplification process as shown in Figure 2. The principle behind the use of overlapping long oligonucleotides or gene fragments in the three round PCR procedure is that the sense strand and anti-sense strands of the nucleotide sequences are complementary at overlapping regions and act as primers after annealing.

Table 1 presents amino acid sequences of the twelve B-cell and nine T-cell epitopes derived from nine stage-specific vaccine candidate antigens of *P. falciparum* used in the development of the protein CDC/NIIMALVAC-1. One universal T-cell epitope from tetanus toxoid is also incorporated. A sequence for the melittin signal peptide, used for enhancement of protein secretion in the baculovirus expression system, is added to the N terminus. A sequence of six histidines is inserted immediately C-terminal to the melittin signal peptide sequence to facilitate purification of expressed recombinant CDC/NIIMALVAC-1 on a nickel column. Corresponding nucleotide sequences for the melittin signal peptide sequence, the six histidine residues and the epitopes from *P. falciparum* are constructed. Restriction enzyme sites *Bam*HI and *Not*1 are designed at the flanking end to facilitate cloning in baculovirus transfer vector. Twelve overlapping single stranded oligonucleotides, each 125-145 nucleotides in length and spanning the entire synthetic gene, are synthesized and the vaccine antigen gene assembled.

The order of different epitopes is chosen such that the final protein has: 1) a random balance of B- and T-cell epitopes; and 2) an overall hydrophilic structure and water solubility. The vaccine antigen gene is ligated into a transfer vector, preferably a

baculovirus transfer vector, such as pBacPAK8, and the recombinants are used to transform host cells such as *Escherichia coli* XL-Blue component cells. For example, lipofectin-mediated transfection and *in vivo* homologous recombination were used to introduce the vaccine antigen gene from pBacPAK8 into *Autographa californica* nuclear polyhedrosis virus (AcNPV, strain E2) at the polyhedrin locus of the genome.

The synthetic gene is cloned, and the recombinant virus containing CDC/NIIMALVAC-1 gene produced and grown in confluent monolayer cultures of an Sf21 insect cell line. The expressed recombinant protein is then purified, preferably using affinity chromatography techniques, and its purity and specificity determined by known methods. Alternatively, the synthetic gene may be employed as a DNA vaccine.

A variety of expression systems may be employed for expression of the recombinant protein. Such expression methods include, but are not limited to the following: bacterial expression systems, including those utilizing *E. coli* and *Bacillus subtilis*; vaccinia virus systems; yeast expression systems; cultured insect and mammalian cells; and other expression systems known to one of ordinary skill in the art.

Purification and Characterization of the Expressed Protein

The expressed protein contains epitopes from the sporozoite stage, liver stage, blood stage and sexual stage (also known as the gametocyte stage) of the malarial parasite *P. falciparum*, as well as a melittin signal peptide, a polyhistidine sequence and an amino acid sequence from tetanus toxoid. Although the antigens (epitopes) listed in Example 1 and Table 1 are the preferred antigens, it is to be understood that other antigens derived from these different stages in the life cycle of *P. falciparum* may be employed and are within the scope of the present invention. It is also to be understood that amino acid substitutions, as described elsewhere herein, may be made for amino acids in the peptide epitopes listed in Table 1, and are within the scope of the present invention. The order of the arrangement of these epitopes may be important in producing an efficacious recombinant protein for use as an antimalarial vaccine against *P. falciparum*. Various arrangements of these epitopes are considered within the scope of the present invention, provided that the arrangements generate an immune response in the recipient to epitopes derived from different stages in the life cycle of *P. falciparum*. A preferred order of these epitopes is presented in Figure 1. The expressed protein, herein referred to as CDC/NIIMALVAC-1, is immunogenic when administered in combination with a carrier and adjuvants to mice and rabbits. Antibodies produced against the recombinant protein CDC/NIIMALVAC-1 recognize epitopes in the sporozoite stage, liver stage, blood stage and sexual stage of the malarial parasite *P. falciparum*.

Antibody Production

The protein is combined with a pharmaceutically acceptable carrier or vehicle to produce a pharmaceutical composition, and is administered to animals for the production of polyclonal antibodies. The preferred animals for antibody production are rabbits and mice. Other animals may be employed for immunization with the recombinant protein. Such animals include, but are not limited to the following; sheep, horses, pigs, donkeys, cows, monkeys and rodents, such as guinea pigs, and rats. Monoclonal antibodies can then be produced using hybridoma technology in accordance with methods well known to those skilled in the art, as taught by Mason *et al.* (Techniques in Immunocytochemistry, Vol. 2, Bullock & Petrusz eds., Academic Press, pp. 175-216, 1983). The antibodies are useful as research or diagnostic reagents or can be used for passive immunization. The pharmaceutical composition used for generation of antibodies may contain an adjuvant.

The antibodies useful as research or diagnostic reagents may be employed for detection of malarial infection in a biological sample, especially infection caused by *P. falciparum.* Such capability is useful for early detection of disease so that the vaccine may be administered to ameliorate disease progression. This capability is also useful for detecting the malarial parasite in the blood, especially blood collected for blood banks, so that malarial transmission through this mode is reduced or eliminated. Other biological samples which can be examined for infection are samples of human and animal livers, and also mosquitoes. Detection may be achieved through the use of ELISA, radioimmunoassay or other assays or methods as commonly known to one of ordinary skill in the art.

The CDC/NIIMALVAC-1 protein may be labeled through commonly known isotopic and non-isotopic methods, including but not limited to the following: radiolabeling, biotin-avidin, fluorescent molecules, chemiluminescent molecules and systems, ferritin, colloidal gold, and other methods known in the art of labeling proteins. The anti-CDC/NIIMALVAC-1 antibodies may be used in combination with labeled CDC/NIIMALVAC-1 protein to detect epitopes in *P. falciparum.*

The anti-CDC/NIIMALVAC-1 antibodies may also be administered directly to humans and animals in a passive immunization paradigm to confer immunity in the recipient to malaria.

Method of Administration

The protein is combined with a pharmaceutically acceptable carrier or vehicle for administration as a vaccine to humans or animals. The terms "pharmaceutically acceptable carrier" or "pharmaceutically acceptable vehicle" are used herein to mean any liquid including, but not limited to, water or saline, a gel, salve, solvent, diluent, fluid ointment base, liposome, micelle, giant micelle, and the like, which is suitable for use in contact

with living animal or human tissue without causing adverse physiological responses, and which does not interact with the other components of the composition in a deleterious manner.

The vaccine formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers. Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets commonly used by one of ordinary skill in the art.

Preferred unit dosage formulations are those containing a dose or unit, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the present invention may include other agents commonly used by one of ordinary skill in the art.

The vaccine may be administered through different routes, such as oral, including buccal and sublingual, rectal, parenteral, aerosol, nasal, intramuscular, subcutaneous, intradermal, and topical. The vaccine may be administered in different forms, including but not limited to solutions, emulsions and suspensions, microspheres, particles, microparticles, nanoparticles, and liposomes. It is expected that from about 1 to 5 dosages may be required per immunization regimen. Initial injections may range from about 1 μg to 1 mg, with a preferred range of about 10 μg to 800 μg, and a more preferred range of from approximately 25 μg to 500 μg. Booster injections may range from 1 μg to 1 mg, with a preferred range of approximately 10 μg to 750 μg, and a more preferred range of about 50 μg to 500 μg.

The volume of administration will vary depending on the route of administration. Intramuscular injections may range from about 0.1 ml to 1.0 ml.

The vaccine may be stored at temperatures of from about 4°C to -100°C. The vaccine may also be stored in a lyophilized state at different temperatures including room temperature. The vaccine may be sterilized through conventional means known to one of ordinary skill in the art. Such means include, but are not limited to filtration, radiation and

heat. The vaccine may also be combined with bacteriostatic agents, such as thimerosal, to inhibit bacterial growth.

Vaccination Schedule

The vaccine of the present invention may be administered to humans, especially individuals traveling to regions where malaria is present, and also to inhabitants of those regions. The optimal time for administration of the vaccine is about one to three months before the initial infection. However, the vaccine may also be administered after initial infection to ameliorate disease progression, or after initial infection to treat the disease.

Adjuvants

A variety of adjuvants known to one of ordinary skill in the art may be administered in conjunction with the protein in the vaccine composition. Such adjuvants include, but are not limited to the following: polymers, co-polymers such as polyoxyethylene-polyoxypropylene copolymers, including block co-polymers; polymer P1005; Freund's complete adjuvant (for animals); Freund's incomplete adjuvant; sorbitan monooleate; squalene; CRL-8300 adjuvant; alum; QS 21, muramyl dipeptide; CpG oligonucleotide motifs and combinations of CpG oligonucleotide motifs; trehalose; bacterial extracts, including mycobacterial extracts; detoxified endotoxins; membrane lipids; or combinations thereof.

It will be appreciated that other embodiments and uses will be apparent to those skilled in the art and that the invention is not limited to these specific illustrative examples.

Example 1

Development, Synthesis and Cloning of the CDC/NIIMALVAC-1 Gene

A recombinant multivalent and multistage vaccine against *P. falciparum* was designed to contain the secretory melittin signal peptide sequence, six histidine (His) residues for purification of the protein, one tetanus toxoid universal T-helper epitope, and 21 immunogenic peptide epitopes (12 B-cell epitopes and 9 T-cell epitopes) from the CSP, Pfg27, SSP-2, LSA-1, MSP-1, MSP-2, AMA-1, EBA-175, and RAP-1 vaccine antigens (see Table 1 and SEQ ID NOS:1-26). These malarial peptide epitopes were obtained from different stages of the life cycle of *P. falciparum* including the following stages: the sporozoite stage (CSP and SSP-2); liver stage (LSA-1); blood stage (MSP-1, MSP-2, AMA-1, EBA-175, and RAP-1); and sexual stage (Pfg27). The peptide epitopes of the CSP, LSA-1, MSP-1, AMA-1, and RAP-1 were identified through vaccine-related field studies in western Kenya, where the genetic diversity of candidate vaccine antigen genes and the characteristics of naturally acquired protective immunity against malaria were being investigated. The epitopes for the Pfg27, MSP-2, EBA-175, and SSP-2 were identified as

involved in conferring protection. The synthetic gene (SEQ ID NO:1) encoding for the recombinant protein was assembled, cloned, and expressed in a baculovirus system. The recombinant gene encoded for a recombinant protein referred to herein as CDC/NIIMALVAC-1 having the sequence of SEQ ID NO:2.

A schematic map shown in Figure 1 provides the locations of epitopes in CDC/NIIMALVAC-1 indicated by the codes corresponding to the codes in Table 1. The rationale of the arrangement of the immune epitopes reflects a random balance of position of B cell and T cell epitopes. Corresponding nucleotide sequences for the melittin signal peptide sequence, the six histidine residues and the epitopes from *P. falciparum* were constructed. The nucleotides shown adjacent to melittin and adjacent to P 599 in Figure 1 are start and stop codons. Restriction enzyme sites *Bam*HI and *Not* I were designed at the flanking end to facilitate cloning in baculovirus transfer vector. Twelve overlapping single stranded oligonucleotides, each 125-145 nucleotides in length and spanning the entire synthetic gene, were synthesized, and the gene encoding for the vaccine antigen was assembled.

The vaccine antigen gene was ligated into a baculovirus transfer vector, pBacPAK8, and the recombinants were used to transform *Escherichia coli* XL-Blue component cells. Lipofectin-mediated transfection and *in vivo* homologous recombination were used to introduce vaccine antigen gene from pBacPAK8 into *Autographa californica* nuclear polyhedrosis virus (AcNPV, strain E2) at the polyhedrin locus of the genome.

The strategy for constructing the gene encoding for CDC/NIIMALVAC-1 described in this example is shown in Figure 2. Twelve long overlapping single-strand oligonucleotides, spanning the full length of gene, were synthesized and assembled through a three step PCR to generate a 1053 base pair gene. The PCR cycles used in each step are indicated in Figure 2.

Table 1

Epitope locati n, amino acid sequences and epitope-specific antibody responses to CDC/NIIMALVAC-1[1]

Antigens[2]	Epitope codes[2]	Sequences and SEQ ID Nos.	Stage[3]	Epitopes	Epitope-specific antibody response[4] in rabbit
Melittin signal peptide	Melittin	MKFLVNVALVFMV VYISYIYAD SEQ ID NO:25	n.a.[5]	n.a.	n.a.
6X His	6X His	HHHHHH SEQ ID NO:26	n.a.	n.a.	n.a.
CSP	P592	KHKKLKQPGDGNP SEQ ID NO:5	S	B	Neg
SSP-2	P547	WSPCSVTCG SEQ ID NO:8	S	B	Neg
CSP	P593	KPKDELDYENDIEK KICKMEKCS SEQ ID NO:6	S	CTL	High
CSP	P594	DIEKKICKMEKCSS VFNVVNS SEQ ID NO:7	S	CS.T3	Medium
MSP-1	P597	NSGCFRHLDEREEC KCLL SEQ ID NO:11	B	B	Low
MSP-1	P598	EDSGSNGKKITCEC TKPDS SEQ ID NO:12	B	B	Neg
LSA-1	P595	KPIVQYDNF SEQ ID NO:9	L	CTL	Medium
CSP	P519	NANPNANPNANP SEQ ID NO:4	S	B	High
AMA-1	P600	DGNCEDIPHVNEFS AIDL SEQ ID NO:16	B	B	High
AMA-1	P601	GNAEKYDKMDEPQ HYGKS SEQ ID NO:17	B	B	Medium

RAP-1	P545	LTPLEELY SEQ ID NO:21	B	B	Medium
LSA-1	P596	KPNDKSLY SEQ ID NO:10	L	CTL	Neg
P2, TT tetanus toxoid	P589	QYIKANSKFIGITEL SEQ ID NO:24	tetanus toxoid	T	Neg
MSP-2	P543	SNTFINNA SEQ ID NO:14	B	B	Neg
MSP-2	P544	GQHGHMHG SEQ ID NO:15	B	B	Neg
EBA-175	P546	NEREDERTLTKEYE DIVLK SEQ ID NO:20	B	B	Low
AMA-1	P603	EFTYMINFGRGQNY WEHPYQKS SEQ ID NO:19	B	T	Low
AMA-1	P602	DQPKQYEQHLTDYE KIKEG SEQ ID NO:18	B	T	Low
Pfg27	P591	KPLDKFGNIYDYHY EH SEQ ID NO:3	G	B	Low
RAP-1	P604	SSPSSTKSSPSNVKS AS SEQ ID NO:22	B	T	Low
RAP-1	P605	LATRLMKKFKAEIR DFF SEQ ID NO:23	B	T	Neg
MSP-1	P599	GISYYEKVLAKYKD DLE SEQ ID NO:13	G	T	Neg

1: Location of immune epitopes in CDC/NIIMALVAC-1 is presented in the first column and indicated by the codes. A melittin signal peptide sequence and 6 histidine residues were incorporated at N-terminus.

2: Abbreviations: CSP, circumsporozoite protein; SSP-2, sporozoite surface protein-2; LSA-1, liver stage antigen-1; MSP-1, merozoite surface protein-1; MSP-2, merozoite surface protein-2; AMA-1, apical membrane antigen-1; EBA-175, erythrocyte binding antigen-175; RAP-1, rhoptry associated protein-1; Pfg27, gametocyte 27 kDa antigen.

3: In the column entitled "Stage", B represents Blood stage, L represents Liver stage, S represents Sporozoite stage, G represents gametocyte stage

4: For epitope-specific antibody response, titers were determined based on the highest dilution of the samples which generate an optical density (OD) greater than the cutoff value

(mean plus 3 standard deviations of preimmunization sera). ODs lower than cutoff at 1:50 dilution were considered a negative response. High response: titers are higher than 1:10,000; medium response: titers are 1:1,000-1:10,000; and low response: titers are 1:50-1:1,000.

5: n.a. not applicable

Example 2

Expression of the Recombinant Protein in a Baculovirus System and Purification of the Protein Antigen

The synthetic gene described in Example 1 was cloned and the recombinant virus containing the CDC/NIIMALVAC-1 gene was produced and grown in confluent monolayer cultures of Sf21 insect cell line, as described by Chatterjee, U. *et al.*, (1996) *Gene* 171:209-213. The synthetic gene is approximately 1053 bp as determined on an agarose gel with corresponding DNA standards run in another lane.

The baculovirus-expressed, recombinant protein was purified from Sf21 cells at 72 hours postinfection using TALON metal affinity resin according to the manufacturer's instructions (Clonetech, Palo Alto, CA). The purity and specificity of the expressed recombinant protein were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The results show that expression of the synthetic gene in the baculovirus expression vector system produced a 42 kD protein referred to herein as CDC/NIIMALVAC-1.

Example 3

Immunization of Rabbits against the Expressed Protein and Demonstration of Immunoreactivity against Different Stages in the Life Cycle of P. falciparum

As described in Example 2, the baculovirus-expressed recombinant protein was purified from cell pellets harvested 72 hours after infection. The expressed protein CDC/NIIMALVAC-1 was purified on nickel affinity column to bind the polyhistidine residues near the N-terminus of the protein using TALON metal affinity resin according to manufacturer's instructions. The purity and specificity of protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described in Example 2. The isolated protein was characterized, using chromatographic techniques, as approximately 42 kD in size.

Six to eight week old mice were immunized intraperitoneally with 50 μg per dose of purified CDC/NIIMALVAC-1 protein in Freund's complete adjuvant. Booster immunizations of mice were 25 μg per dose of purified CDC/NIIMALVAC-1 protein in Freund's incomplete adjuvant. A total of 4 immunizations were given to each mouse at 3-week intervals (i.e., at weeks 0, 3, 6, and 9), blood was removed and sera were collected 7 to 10 days after each immunization until weeks 33-51. Sera were stored at -20°C. The sera from mice were used in the antibody and immunofluorescence (IFA) assays.

Four-month-old female New Zealand white rabbits (Jackson Laboratory, Bar Harbor, ME) were immunized intramuscularly with 100 μg per dose of purified CDC/NIIMALVAC-1 protein in vehicle. One rabbit was immunized with the protein in Freund's adjuvant (rabbit 787). Another rabbit was immunized with the protein in a potentially human-usable adjuvant, specifically the nonionic block copolymer P1005 in water-in-oil emulsion (rabbit 789). Another rabbit was immunized with the protein in the human usable adjuvant, aluminum hydroxide (rabbit 1015). A total of 4 immunizations were given to each rabbit at 3-week intervals (i.e., at weeks 0, 3, 6, and 9), blood was removed and sera were collected 7 to 10 days after each immunization until weeks 33-51. Sera were stored at -20°C. The sera from rabbits were used in the antibody and IFA assays.

Sera collected from the blood of each rabbit during weeks 10 to 16 were pooled and total IgGs were purified using ammonium sulfate (Sigma Chemical Co., St. Louis, MO) precipitation followed by DEAE (Pierce, Rockford, IL) batch purification according to known methods as taught by Hollingdale, M.R. *et al.*, (1984) *J. Immunol.* 132, 909-913. Following dialysis against PBS, the purified antibodies were used for immunoelectron microscopy, antibody affinity testing, and in an *in vitro* protection assay

Serum antibody titers against the vaccine antigen and individual peptide epitopes were quantitated by ELISA. Microtiter plates were coated with the vaccine antigen or peptides in borate buffer solution (BBS) overnight at 4°C and then blocked with BBS containing 5% nonfat lyophilized milk. The plates were washed four times with sodium phosphate-buffered saline (PBS, pH 7.4) containing 0.5 M NaCl, 0.5% bovine serum albumin, 0.0005% Tween 20, and 0.05% thimerosal (PBS-T). The rabbit sera were diluted serially in PBS-T containing 1.5% nonfat milk, added into microtiter plates, and incubated at room temperature for 1 hour. The unbound antibodies were removed by four washes with PBS-T. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit antibodies. The secondary antibody was allowed to bind for 1 hour, the wells were washed with PBS-T, 100 μl of 3,3',5,5'-tetramethylbenzidine was added, and 10 minutes later the reaction was stopped with 1M phosphoric acid. The plates were read at an absorbance of 450 nm. Responses against sporozoites, asexual blood-stage parasites, and gametocytes were determined by indirect immunofluorescence (IFA). All immunized rabbits had high and comparable IFA titers against sporozoites (1:3,200). IFA titers against infected erythrocytes were in the range of 1:50-1:400, with the highest titers observed in the rabbit receiving copolymer as adjuvant. The reactivity with gametocytes showed IFA titers of 1:25 to 1:100.

The CDC/NIIMALVAC-1 protein was found to be immunogenic as confirmed by its ability to elicit immune responses against both CDC/NIIMALVAC-1 and different

stages in the life cycle of the *P. falciparum* parasite. Antibody was purified from the rabbit immunized with CDC/NIIMALVAC-1 in copolymer adjuvant.

The immunoreactivity of this purified rabbit antiserum against antigens present in the different stages of the life cycle of the malarial parasite, *P. falciparum*, was evaluated using ultrastructural immunocytochemistry. To study ultrastructural localization of antibody reactivities with various stages of *P. falciparum*, sporozoite, exoerythrocytic (EE)-infected hepatocyte, gametocyte stage III-IV, and asexual blood stage parasites were chosen for immunoelectron microscopy. Briefly, sections were incubated for 24 hours at 4°C with antibody diluted 1:800 for determination of reactivity with sporozoites, or with antibody diluted 1:200 for determination of reactivities with other stages of parasites. This step was followed by 1 hour incubation at 25°C with gold-labeled, goat anti-rabbit IgG antibody. Method specificity was confirmed by incubating control sections with preimmune rabbit serum instead of the primary antibody, with the colloidal gold probe, or with colloidal gold alone. Immunoreactivities of antibodies with parasites were examined in a Zeiss CEM902 electron microscope as taught by Aikawa, M. & Atkinsen, C.T. (1990) *Adv. Parasitol.* 29, 151-214.

Immunoelectron micrographs of different stages in the life cycle of the *P. falciparum* parasite demonstrated that the rabbit antiserum from rabbit 789, which received copolymer as adjuvant, contained antibodies which were immunoreactive to the sporozoite stage, blood stage, gametocyte stage III-IV, and asexual blood stage. Gold particles were found on the surface and in the cytoplasm of the sporozoite, in the parasitophorous vacuole membrane (PVM) and cytoplasm of blood stage, in the cytoplasm in the gametocyte stage III-IV, in rhoptry and surface of merozoite of the blood stage and in the cytoplasm of trophozoite of the blood stage. There was no observed immunoreactivity against these stages using purified IgG from a normal control rabbit instead of the purified immune serum from the immunized rabbit.

Immunogenicity of the Multicomponent P. falciparum *Vaccine*

Antibody titers against the vaccine antigen CDC/NIIMALVAC-1 and peptides complementary to 22 immune epitopes (Table 1) were measured by ELISA after each immunization until week 51 for rabbit 787 (Freund's adjuvant), week 39 for rabbit 789 (copolymer adjuvant), and week 33 for rabbit 1015 (alum adjuvant). Figure 3 shows that the vaccine antigen induced high titer and prolonged antibody responses against the vaccine in rabbits immunized with different adjuvants (1/3,276,800 titer for rabbit 787, 1/819,200 titer for rabbit 789, and 1/204,800 titer for rabbit 1015, after the fourth immunization). Overall, the rabbit receiving Freund's adjuvant had higher antibody levels to the vaccine antigen compared to those receiving copolymer or alum adjuvants. The

antibody responses reached maximal levels after the fourth immunization, remained at high levels until weeks 14 to 16, and then decreased 10- to 15-fold between weeks 33 to 51.

Analysis of epitope-specific antibody responses by ELISA showed that the antibodies recognized both B-cell (7 out of 12) and T-cell (6 out of 10) epitopes in this vaccine. Among the epitope-specific antibody responses, antibody levels against B-cell epitopes of CSP (P519), AMA-1 (P600), and CTL epitope of CSP (P593), were significantly higher than antibody levels to other epitopes. Like antibody responses to the whole vaccine antigen, the antibody titer against these epitopes was also higher in rabbits receiving vaccine with Freund's adjuvant and copolymer adjuvant than those receiving vaccine with alum as adjuvant. Medium-to-low antibody responses to other epitopes were observed. Medium-level antibody responses were observed against the B-cell epitopes of AMA-1 and RAP-1 (P601 and P545), and T-cell epitopes of CSP and LSA-1 (P594 and P595). Low-level antibody responses were detected against B-cell epitopes of EBA-175, MSP-1 and Pfg27 (P546, P597, and P591), and T-cell epitopes of AMA-1 and RAP-1 (P602, P603 and P604).

The binding affinities of the vaccine-elicited antibodies to the vaccine antigen itself were investigated in a Biacore assay employing a surface-plasmon resonance detector (Biacore, Inc., Piscataway, NJ). Purified vaccine antigen was covalently immobilized in a "C1" (short-chain carboxymethyl-dextran) sensor cell by standard carbodiimide/N-hydroxysuccinimide methods. Mobile-phase analyte consisted of purified IgG preparations diluted to 50 μg of protein per ml in 10 mM HEPES buffer, pH 7.4, plus 150 mM NaCl. After establishing a stable baseline signal with buffer, the association reaction was initiated by switching to the analyte stream, and the resonance signal followed in time, as taught by Wohlhueter, R.M. *et al.*, (1994) *J. Immunol.* 153, 181-189. A flow rate of 5 μl/min was used throughout. Association rate curves observed with different IgG preparations were adjusted to a common baseline and superimposed. Initial velocities of association of the high-affinity components were estimated by measuring the initial linear slopes of the curves (e.g., in the interval of 430 to 500 seconds on the abscissa of Figure 4). Antigen-antibody binding showed multiple kinetic components distinguishable into fast (450-500 seconds) and slow (800-1400 seconds) components (Fig. 4). Quantitatively, total IgG from the rabbit (#789) immunized in the presence of copolymer adjuvant contained much higher levels of high affinity antibodies. This conclusion was also supported by the biological analysis described below.

Example 4

Use of Growth Inhibition Assays (GIA) and Antibody Dependent Cellular Inhibition Assays (ADCI) for Determining Growth Inhibitory Effects of Purified Antibodies

Antibodies were purified from rabbits immunized with the CDC/NIIMALVAC-1 protein in Freund's adjuvant (R-Freund's), in copolymer adjuvant (R-copolymer), and in alum adjuvant (R-alum-1 and R-alum-2), respectively. The results of *in vitro* experiments, performed to test the protective effects of antibodies with (antibody dependent cellular inhibition, ADCI) or without human monocytes (growth inhibition assay, GIA), showed that the antibodies against CDC/NIIMALVAC-1 inhibited parasite growth (Table 2B). The inhibitory effects were directly related to the concentration of antibodies (Table 2B).

Inhibition of sporozoite invasion (ISI) assays were conducted to determine the inhibitory effects of antibodies as previously described by Hollingdale, M.R. *et al.*, (1984) *J. Immunol.* 132, 909-913. Briefly, the purified antibodies were added at two different final concentrations (25 and 50 μg/ml) into the HepG2-A16 hepatoma cells, and then about 30,000 *P. falciparum* sporozoites were added. The cells were incubated at 37°C in 5% CO_2 for 3 hours, rinsed two times with phosphate-buffered saline (PBS), and fixed with methanol. Sporozoites that had entered hepatoma cells were visualized by immunocytochemical staining with a monoclonal antibody to *P. falciparum* sporozoites (NSF1), peroxidase-conjugated, goat anti-mouse immunoglobulin and the substrate 3,3-diaminobenzidine. All cultures were done in triplicate and the numbers of sporozoites that had invaded the hepatoma cells were determined by light microscopy.

Antibody dependent cellular inhibition (ADCI) assays were carried out using described methods (Bouharoun-Tayoun, H. *et al.*, (1995) *J. Exp.Med.* 182, 409-418; Shi, Y.P. *et al.*, (1999) *Am. J. Trop. Med. Hyg.* 60(1), 135-141). Briefly, the purified antibodies were added at three different final concentrations (12.5, 25, and 50 μg/ml) into FC27 strain blood-stage parasite cultures (0.3% parasitemia with 60% schizonts, and 1% hematocrit), along with 80,000 rhIFN-gamma (100 ng/ml)-activated human monocytes. The cell cultures were incubated at 37°C in a mixed gas containing 5% O_2, 5% CO_2, and 90% N_2 for 72 hours, with medium and antibody replacement every 24 hours. Parasites were stained with the vital dye hydroethidine (HE) and parasitemias were determined by a flow cytometry-based parasite enumerating procedure using FACScan. Transmission-blocking assays were performed by membrane feeding assays as taught by Wizel B. & Kumar, N. (1991) *Proc. Natl. Acad. Sci.* USA 88, 9533-9536. In this assay, *P. falciparum* (3D7) gametocytes were used to infect *An. stephensi* mosquitoes. Various IgG preparations were tested at the final concentration of 125 to 500 μg/ml.

Determination of in vitro antiparasite activity of vaccine-elicited antibodies

Antibodies from rabbits immunized with the vaccine in different adjuvants strongly inhibited sporozoite invasion of HepG2-A16 cells (Table 2A). Antibodies from the rabbit which received the vaccine in block copolymer adjuvant almost completely inhibited sporozoite invasion (98% inhibition) at an antibody concentration of 50 μg/ml. This level of inhibition was comparable with the inhibition observed using a positive monoclonal antibody control. Antibodies elicited against the vaccine in the presence of alum or Freund's adjuvants also inhibited invasion of sporozoites, although at lower levels. At 25 μg/ml, antibody-mediated inhibition was again most prominent in the case of antibodies from rabbits vaccinated with recombinant protein plus block copolymer adjuvant.

ADCI experiments showed that the vaccine-elicited antibodies had significant inhibitory effects on *in vitro* growth of blood-stage parasites in the presence of monocytes. No growth inhibitory effects in the absence of monocytes were observed. The most striking ADCI activity was mediated by antibodies from the rabbit which received the vaccine co-polymer formulation (73% inhibition); lower activity was observed in those animals receiving vaccination in Freund's adjuvant (70%), and alum adjuvant (67%) (Table 2B). A concentration-dependent, antibody inhibition of growth was observed in the ADCI assay (Table 2B). As compared with the ADCI activity with the rabbit antivaccine antibodies, the activity of purified IgGs from Kenyan adults, who were clinically immune to malaria, was much lower (between 15%-29%). As compared with the sporozoite and blood-stage inhibitory activities, the results of transmission blocking assays did not reveal any significant inhibition.

Example 5

Test of Immunogenicity and Protective Efficacy of the Multivalent Vaccine in a Monkey Model System

Monkeys received about 100 μg of the vaccine described in Example 3 per animal for initial immunization and approximately 200 μg per animal for booster injection. Animals were immunized with vaccine alone, and also in the presence of one of the following different adjuvants: alum; copolymer P1005 in saline; copolymer P1005 in water-in-oil; QS21 (Acquila Biopharmaceuticals, Boston, MA); Freund's complete adjuvant followed by Freund's incomplete adjuvant; and SBAS2 (Smith-Kline-Beechum, Belgium). The non-immunized group served as a control. Seven *A. nancymai* monkeys were assigned per treatment group except for the control group of 6 animals (n=55). After 4 immunizations, animals are challenged with live parasites and the course of parasitemia is monitored in immunized and non-immunized animals using techniques known to one of ordinary skill in the art. Blood samples are collected periodically throughout the trial to

Table 2: Evaluation of in vitro anti-parasitic activities of CDC/NIIMAL VAC-1 elicited antibodies

A: Inhibition of sporozoite invasion (ISI)

Antibodies	Antibody concentrations (ug/ml)			
	50		25	
	Invasion number1	%inhibition2	Invasion number1	%inhibition2
Preimmune3	131(10)	0	112 (7)	0
Freund	19(3)	85	29 (3)	74
Copolymer	3(2)	98	10 (3)	91
Alum 1015	15(2)	89	23 (4)	79
Positive control4	ND	ND	2(2)	98

1: Mean number of *P.falciparum* sporozoites that entered triplicate cell culture with standard deviation (SD) given in parenthesis.
2: % inhibition expressed relative to pre-immune control culture (0%), which was calculated as follow : 100x[1-(mean number of invaded sporozoites in test culture / mean number of invaded sporozoites in control culture)].
3: Preimmune, antibodies purified from preimmune rabbits used as negative control; Freund, antibodies purified from rabbit receiving Freund's as adjuvant; Copolymer, antibodies purified from rabbit receiving nonionic block copolymer P1005 as adjuvant; Alum 1015, antibodies purified from rabbit receiving aluminum hydroxide as adjuvant.
4: Monoclonal antibody against sporozoites (NFS1) used as positive control .
ND: not done.

B: Growth inhibition of the blood-stage parasite in the presence of monocytes (ADCI)

Antibodies	Antibody concentrations (ug/ml)								
	50			25			12.5		
	Parasitemia			Parasitemia			Parasitemia		
	No monocyte1	Monocyte1	%Inhibition2	No monocyte1	Monocyte1	%Inhibition2	No monocyte1	Monocyte1	%Inhibition2
Preimmune3	2.56(0.07)	1.64(0.1)	0	3.39 (0.09)	2.25 (0.065)	0	3.75 (0.13)	2.48 (0.105)	0
Freund	3.33(0.07)	0.65(0.025)	70	3.49 (0.04)	1.77 (0.125)	23	3.56 (0.025)	2.19 (0.095)	7
Copolymer	2.85(0.09)	0.50(0.025)	73	3.10 (0.12)	1.43 (0.11)	30	3.46 (0.01)	1.68 (0.06)	26
Alum 1015	2.97(0.14)	0.63(0.11)	67	3.33 (0.05)	1.10 (0.05)	50	3.65 (0.125)	1.40 (0.17)	42
Positive control4	3.17(0.1)	1.17(0.035)	29		ND			ND	

1: Mean of parasitemias in duplicate cell culture with standard deviation (SD) given in parenthesis.
2: % inhibition expressed relative to preimmune control culture (0%) and taking into account the possible inhibition induced by monocyte and antibody alone , which was calculated as follow: 100x[1-((mean parasitemia with test IgG and monocyte / mean parasitemia with test IgG and without monocyte)/(mean parasitemia with control IgG and monocyte/ mean parasitemia with control IgG and without monocyte))].
3: Preimmune, antibodies purified from preimmune rabbits used as negative control; Freund, antibodies purified from rabbit receiving Freund's as adjuvant; Copolymer, antibodies purified from rabbit receiving nonionic block copolymer P1005 as adjuvant; Alum 1015, antibodies purified from rabbit receiving aluminum hydroxide as adjuvant.
4: Purified antibody from Kenyan immune adult (1mg/ml) used as positive control.
ND: not done.

evaluate humoral and cellular immune responses to CDC/NIIMALVAC-1 and to various peptides contained within CDC/NIIMALVAC-1.

Example 6

Determination of Immunogenicity of CDC/NIIMALVAC-1 in Inbred Mice

Six- to eight-week old female, inbred strains of mice, C57BL/6 (H-2^b), B10.BR (H-2^k), and B10.D2 (H-2^d), were immunized subcutaneously with about 10 μg of purified CDC/NIIMALVAC-1 with alum adjuvant, or with a nonionic copolymer adjuvant P1005 in a water-in-oil emulsion. Control mice received only either adjuvant. A booster dose (10 μg/mouse) was given 2 weeks after the primary immunization. Mice were bled at different time intervals, and cellular and humoral immune responses were determined according to published techniques (Lal, A.A., *et al.*, (1996) *Infect. Immun.* 64, 1054-1059; Coligan, J.E., *et al.*, Current protocols in immunology (1996), Vol. 1, pp. 2.1.2-2.1.6, pp. 3.12.1-3.1.4, pp. 6.8.1-6.8.3, and Vol. 2, pp. 7.10.1-7.10.6, National Institutes of Health, John Wiley & Sons, Inc.).

B10.BR mice, irrespective of the adjuvant used, generated highest antibody titers after two immunizations. In the C57BL/6 mice, copolymer adjuvant induced the highest antibody titer. B10.D2 mice were low responders, but the copolymer adjuvant induced higher antibody responses than alum (Fig. 5). These results demonstrate that antibody production against CDC/NIIMALVAC–1 is influenced by both mouse H-2 genetic background and the adjuvant used for immunization.

The best proliferative response was observed in B10.BR mice followed by C57BL/6 mice and B10.D2 mice. The stimulation index (SI), which is a measurement of proliferative responses, in the adjuvant control group of mice was less than 2, indicating that the vaccine-induced response is specific as taught by Lal, A.A. *et al.*, (1996) *Infect. Immun.* 64, 1054-1059. The peak proliferative response was observed on day 21, and the response declined at the subsequent time points (Table 3). IFN-γ levels were specifically elevated in immunized mice when compared to adjuvant control mice (Table 4). This IFN-γ response, unlike the proliferative response, was similar in all three strains of mice.

Example 7

Immunogenicity of CDC/NIIMALVAC-1 in Outbred Mice Using Different Adjuvant-Vaccine Formulations

Outbred ICR mice were immunized subcutaneously with 10 μg of CDC/NIIMALVAC-1 (2 injections total) at two week intervals in the presence of CpG oligonucleotides, QS21, copolymer and alum adjuvants. Control mice were given adjuvant alone. Four days after the second immunization, spleens were collected from two

Table 3. Proliferative response to CDC/NIIMALVAC-1 in immunized inbred mice

Mice	Day of assay[1]	Alum control[2]	Alum + vaccine	Copolymer control	Copolymer + vaccine
C57BL/6	21	0.428	4.12	NT[3]	2
	34	1.2	3.21	1.39	2.07
	48	1.2	1.6	0.83	0.35
B10.BR	21	1.11	8	1.15	6.74
	34	1.97	4.92	0.97	3.64
	48	1.59	5.07	1.68	4.08
B10.D2	21	0.29	1.2	0.59	3.4
	34	1.02	1.98	1.09	1.9
	48	0.66	3.33	0.32	2.24

1: Indicates the time at which after first immunization the spleens were obtained for in vitro proliferative assay. Spleens from two immunized or control mice were pooled and proliferative assays were done as described in the specification.
2: The value represents stimulation index. A stimulation index of >2 was considered a positive response.
3: Not tested

Table 4. IFN-γ response to CDC/NIIMALVAC-1 in immunized inbred mice

Mice	Day of assay	Alum control	Alum + vaccine	Copolymer control	Copolymer + vaccine
C57BL/6	21	16	0.48	74	11
	34	0	743	0	1641
	48	247	1449	0	1413
B10.BR	21	4	0.13	295	65
	34	168	2614	0	1745
	48	9	967	0	1633
B10.D2	21	14	10	0	0
	34	318	925	0	1051
	48	10	1320	0	2508

Spleens from two mice were pooled and cultured at $1X10^6$ cell/well in 48 well plate. The cultures were stimulated with 1μg/ml of CDC/NIIMALVAC-1 and supernatants were collected 48 hours later. The values are expressed as pg/ml of IFN-γ in the culture supernatant and these values are represented after the subtraction of background cytokine levels in unstimulated control cultures.

immunized mice and one control mouse. The T cell responses to the vaccine and the synthetic peptides corresponding to the epitopes included in the vaccine were evaluated using the *in vitro* proliferative assay. The results are presented as stimulation index (SI). SI values of greater than 2 are considered positive. Total IgG antibody levels against the vaccine antigen in mice at day 45 and day 60 were determined using ELISA.

The mice immunized with the QS21 and the copolymer adjuvant-vaccine formulations displayed elevated total IgG antibody titers 2 weeks after the third immunization, and maintained high total IgG antibody titers 5 weeks after the third immunization. All mice immunized with different adjuvant-vaccine formulations were able to induce a strong proliferative response to CDC/NIIMALVAC-1. These proliferative responses, observed in an *in vitro* stimulation assay, were dependent on the antigen concentration. Among the four adjuvants, alum, CpG, and QS21-vaccine formulations induced a proliferative response to some of the individual epitopes (Pfg27, CSP, MSP-1, AMA-1, EBA-175 and RAP-1) (Table 5). Control mice immunized with adjuvant alone showed no antibody response or proliferative response to CDC/NIIMALVAC-1, indicating that the humoral and cellular immune responses are vaccine specific. These results indicate that the T cells of mice immunized with CDC/NIIMALVAC-1 recognize individual peptide epitopes derived from different stages in the life cycle of *P. falciparum* and contained within the vaccine. These data also suggest that the vaccine is effective in conferring protection to the different stages in the life cycle of *P. falciparum*.

Example 8

Determination of Immune Responses to CDC/NIIMALVAC-1 in Individuals Naturally Exposed to Malaria

Lymphocyte proliferation, cytokine, and antibody responses to CDC/NIIMALVAC-1 were tested in nonimmune children and clinically immune adults from western Kenya, a malaria holoendemic area. Finger prick samples of heparinized blood were used in this study. The serum samples were used in determining the antibody response against the vaccine antigen and/or peptides in the vaccine antigen using ELISA methodology. In the case of T-cell proliferation assays, peripheral blood mononuclear cells (PBMCs) from these individuals were used. The PBMCs were cultured in the presence of vaccine antigen, CDC/NIIMALVAC-1. The T-cell proliferation was measured quantitatively and the cell culture supernatant was used for measuring cytokine levels using published techniques (Lal, A.A., *et al.*, (1996) *Infect. Immun.* 64, 1054-1059; Coligan, J.E., *et al.*, Current protocols in immunology (1996), Vol. 1, pp. 2.1.2-2.1.6, pp. 3.12.1-3.1.4, pp. 6.8.1-6.8.3, and Vol. 2, pp. 7.10.1-7.10.6, National Institutes of Health, John Wiley & Sons, Inc.).

Table 5: Epitope-specific proliferative response in outbred mice immunized with different adjuvant-vaccine formulations

Antigen	Stage		Alum	V/Alum	V/Alum	CpG	V/CpG	V/CpG	QS21	V/QS21	V/QS21	CoP	V/CoP	V/CoP
Pfg27	Gametocyte	591		XXXXX										
CSP	Sporozoite	519												
CSP	Sporozoite	593					XXXXX							
CSP	Sporozoite	594												
SSP-2	Sporozoite	547												
LSA-1	Liver Stage	595												
LSA-1	Liver Stage	596												
MSP-1	Blood stage	597					XXXXX							
MSP-1	Blood stage	598												
MSP-1	Blood stage	599			XXXXX					XXXXX				
MSP-2	Blood stage	543												
MSP-2	Blood stage	544												
AMA-1	Blood stage	600												
AMA-1	Blood stage	601												
AMA-1	Blood stage	602		XXXXX										
AMA-1	Blood stage	603												
EBA-175	Blood stage	546		XXXXX			XXXXX			XXXXX	XXXXX			
RAP-1	Blood stage	545					XXXXX							
RAP-1	Blood stage	604												
RAP-1	Blood stage	605		XXXXX			XXXXX							
P2	Tetanus Toxoid	589												

: Negative response

XXXXX : Positive response

PBMCs from 76 infants age less than 12 months, and 59 adults from western Kenya, were examined for proliferative responses to CDC/NIIMALVAC-1. The individuals with a stimulation index (SI) value greater than 2.5 were considered positive responders. The results showed no difference in the rate of positive responders and the SI levels between infants and adults. However, PBMCs from infants required much higher concentrations of CDC/NIIMALVAC-1 for *in vitro* stimulation compared to the PBMCs from clinically immune adults. Among 37 infant positive responders, 73% displayed a high proliferative response when using antigen concentrations between 0.5 and 1 μg/ml, whereas 19% required 2.5 to 5 μg/ml of antigen. In contrast, 79% of adult positive responders (n=28) required an antigen concentration of from about 0.001 to 0.1 μg/ml for *in vitro* stimulation. These results indicate that malaria-specific immune activation is much higher in adults than in infants in a malaria holoendemic area.

All patents, publications and abstracts cited above are incorporated herein by reference in their entirety.

It should be understood that the foregoing relates only to preferred embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the present invention as defined in the following claims.

CLAIMS

What is claimed is:

1. A recombinant protein comprising peptides that contain antigenic epitopes from two or more stages in a life cycle of *Plasmodium falciparum.*

2. The protein of Claim 1, wherein the peptides are selected from the group consisting of SEQ ID NOS:3-25, fragments thereof, combinations thereof, and conservative substitutions thereof.

3. The protein of Claim 1, comprising the amino acid sequence of SEQ ID NO:2, fragments thereof, or conservative substitutions thereof.

4. The protein of Claim 1, further comprising a signal peptide, polyhistidine, and a T-cell helper epitope.

5. The protein of Claim 1, wherein the stages are selected from the group consisting of sporozoite stage, liver stage, blood stage and sexual stage.

6. The protein of Claim 1, encoded by the nucleotide sequence of SEQ ID NO:1, fragments thereof, or conservative substitutions thereof.

7. A method of enhancing an immune response of a human or animal to *Plasmodium falciparum* comprising administration to the human or animal of an effective amount of a protein composition comprising a recombinant protein in a pharmaceutically acceptable carrier, the recombinant protein comprising peptides that contain antigenic epitopes from two or more stages in a life cycle of *Plasmodium falciparum.*

8. A protein composition comprising a recombinant protein in a pharmaceutically acceptable carrier, the recombinant protein comprising peptides that contain antigenic epitopes from two or more stages in a life cycle of *Plasmodium falciparum*, wherein the protein composition is immunogenic when administered to a human or to a nonhuman animal.

9. Antibodies immunoreactive to a recombinant protein comprising peptides that contain antigenic epitopes from two or more stages in a life cycle of *Plasmodium falciparum.*

10. Use of the antibodies of Claim 9 for detection or measurement of peptides derived from two or more stages in the life cycle of *Plasmodium falciparum.*

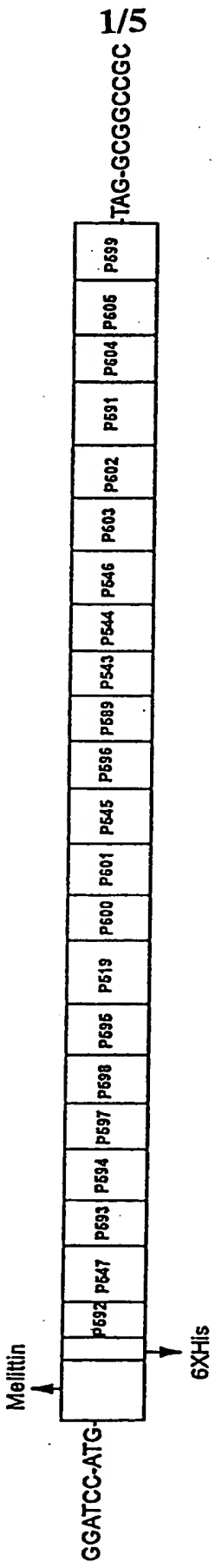
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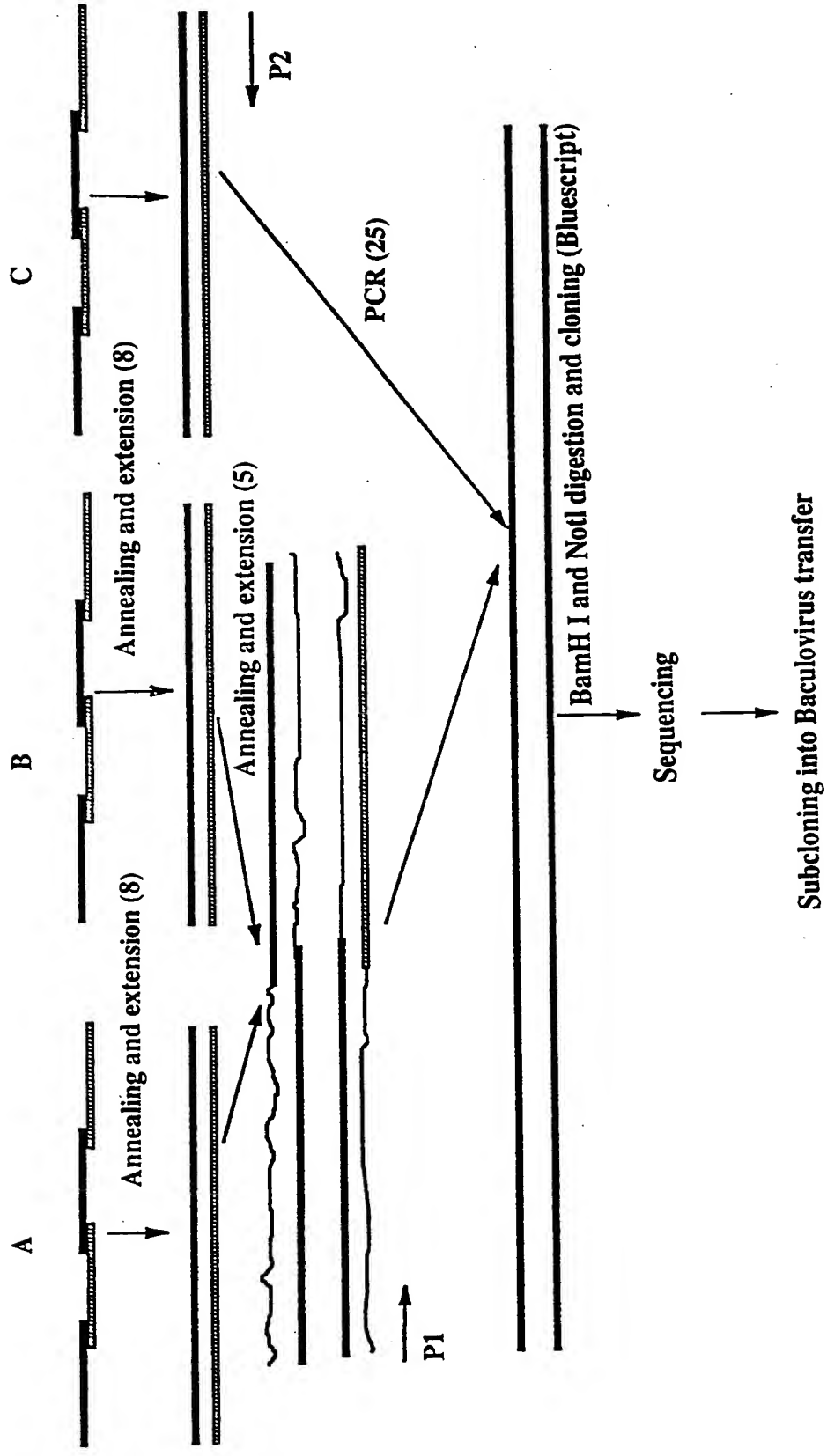
FIG. 1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B
C
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Annealing and extension (5)
Annealing and extension (8)
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P2
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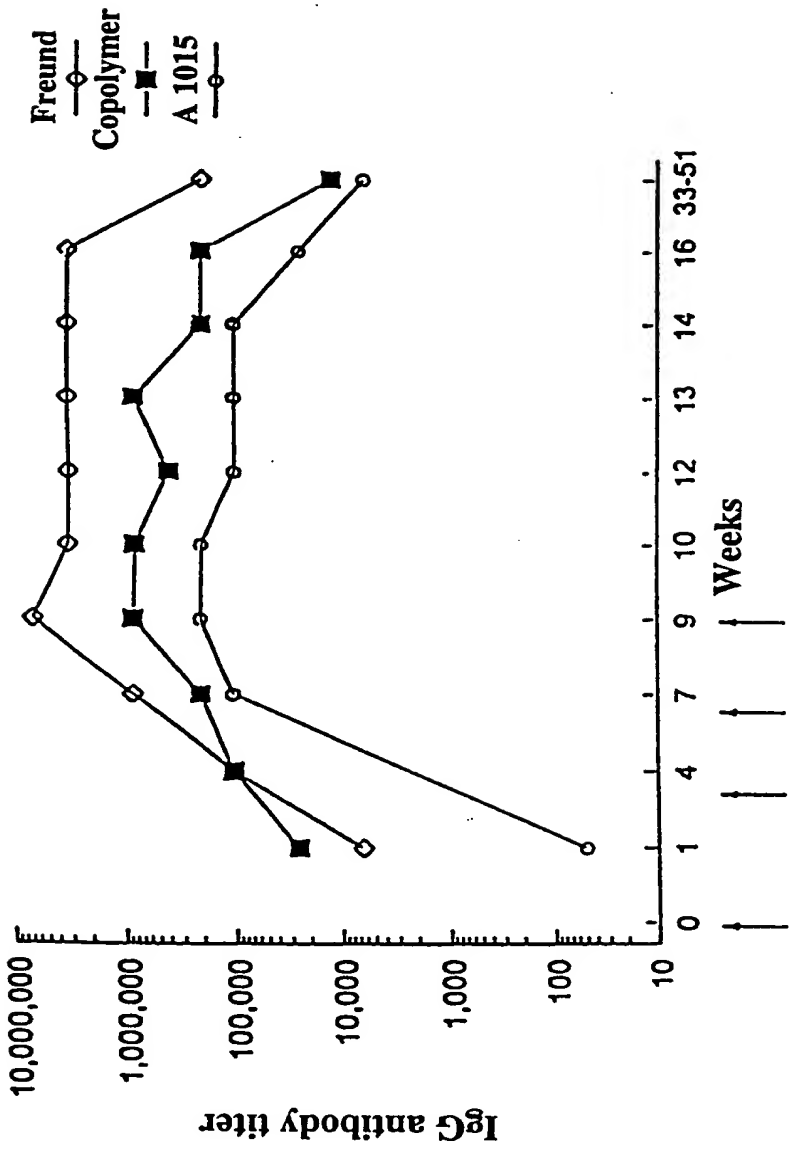
Sequencing
Subcloning into Baculovirus transfer

FIG. 2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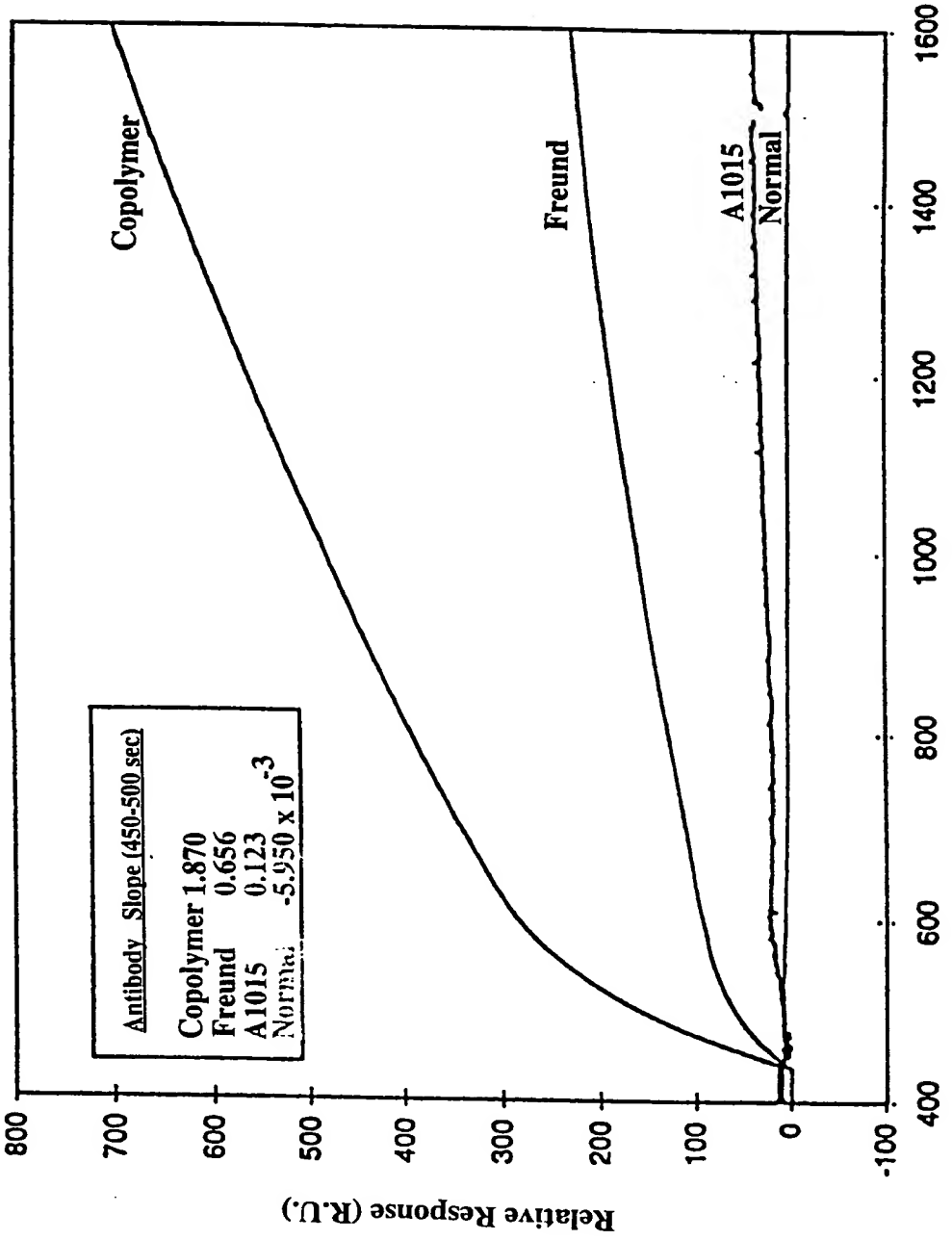
7
9
10
12
13
14
16
33-51
Weeks

FIG. 3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Relative Response (R.U.)
Time (sec)
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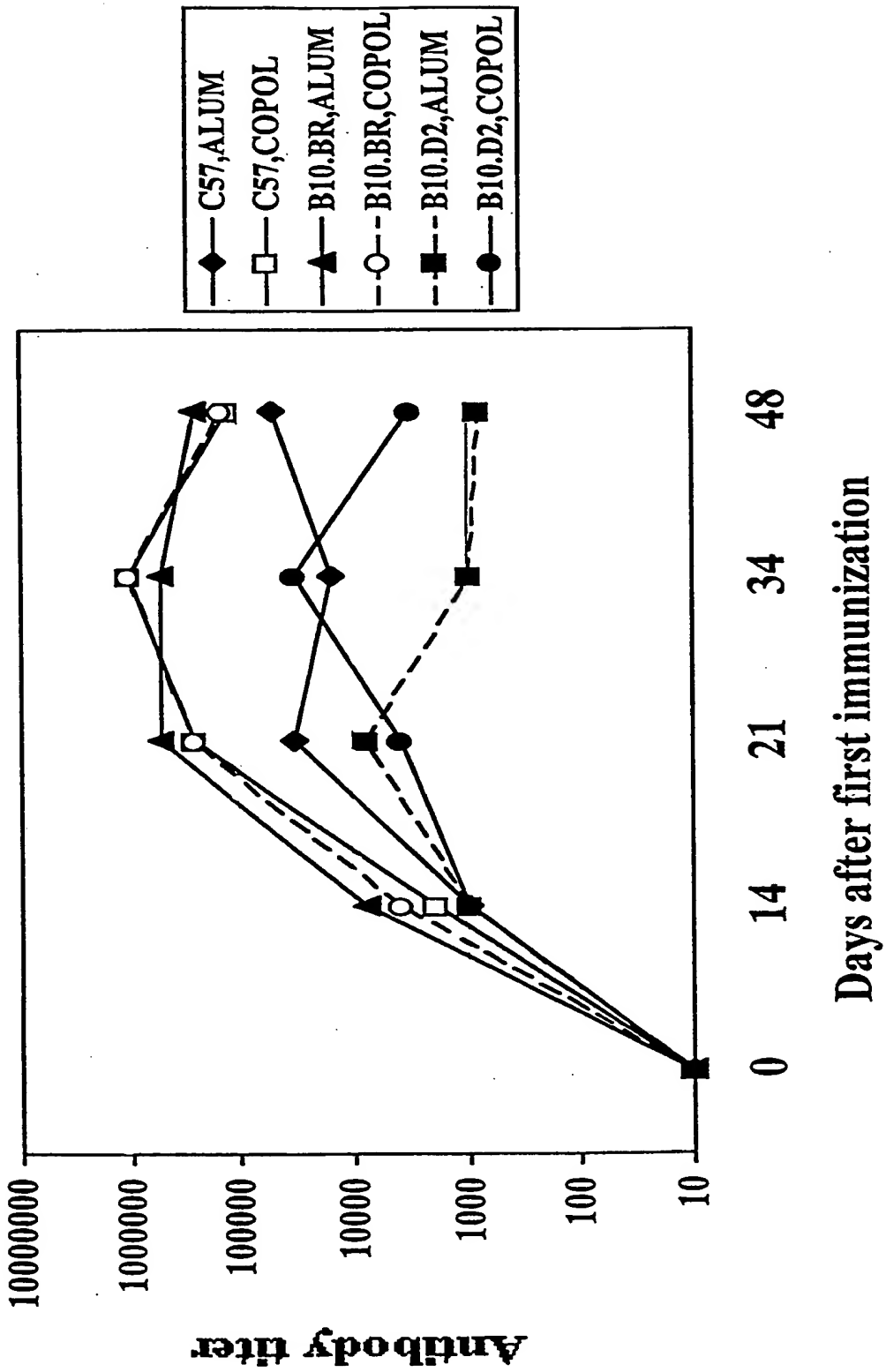
FIG. 4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C57,COPOL
B10.BR,ALUM
B10.BR,COPOL
B10.D2,ALUM
B10.D2,COPOL
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1000
100
10
Antibody titer
0
14
21
34
48
Days after first immunization

FIG. 5

SEQUENCE LISTING

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Leu Lys Gln Pro Gly Asp Gly Asn Pro Trp Ser Pro Cys Ser Val Thr
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Cys Gly Lys Pro Lys Asp Glu Leu Asp Tyr Glu Asn Asp Ile Glu Lys
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Lys Ile Cys Lys Met Glu Lys Cys Ser Ser Val Phe Asn Val Val Asn
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Glu Lys Tyr Asp Lys Met Asp Glu Pro Gln His Tyr Gly Lys Ser Leu
                165                 170                 175

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         35                  40                  45

Cys Gly Lys Pro Lys Asp Glu Leu Asp Tyr Glu Asn Asp Ile Glu Lys
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Lys Ile Cys Lys Met Glu Lys Cys Ser Ser Val Phe Asn Val Val Asn
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Ser Asn Ser Gly Cys Phe Arg His Leu Asp Glu Arg Glu Glu Cys Lys
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Cys Leu Leu Glu Asp Ser Gly Ser Asn Gly Lys Lys Ile Thr Cys Glu
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Cys Thr Lys Pro Asp Ser Lys Pro Ile Val Gln Tyr Asp Asn Phe Asn
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Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asp Gly Asn Cys Glu
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Asp Ile Pro His Val Asn Glu Phe Ser Ala Ile Asp Leu Gly Asn Ala
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Glu Lys Tyr Asp Lys Met Asp Glu Pro Gln His Tyr Gly Lys Ser Leu
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Thr Pro Leu Glu Glu Leu Tyr Lys Pro Asn Asp Lys Ser Leu Tyr Gln
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Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Ser Asn
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Thr Phe Ile Asn Asn Ala Gly Gln His Gly His Met His Gly Asn Glu
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Leu Thr Asp Tyr Glu Lys Ile Lys Glu Gly Lys Pro Leu Asp Lys Phe
        275                 280                 285

Gly Asn Ile Tyr Asp Tyr His Tyr Glu His Ser Ser Pro Ser Ser Thr
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Lys Ser Ser Ser Pro Ser Asn Val Lys Ser Ala Ser Leu Ala Thr Arg
305                 310                 315                 320

Leu Met Lys Lys Phe Lys Ala Glu Ile Arg Asp Phe Phe Gly Ile Ser
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Tyr Tyr Glu Lys Val Leu Ala Lys Tyr Lys Asp Asp Leu Glu
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Ser Tyr Ile Tyr Ala Asp
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<210> 26
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<223> Description of Artificial Sequence: synthetic

<400> 26
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INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/US 99/18869

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/30 C12N15/62 C07K14/445 C07K16/20 A61K39/015
A61P33/06 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K A61P G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GILBERT ET AL.: "A protein particle vaccine containing multiple malaria epitopes" NATURE BIOTECHNOLOGY, vol. 15, November 1997 (1997-11), pages 1280-1284, XP002128163 page 1280, column 2 -page 1283, column 1; figures 1-4; table 1 ---	1-10
A	US 5 766 597 A (DE TAISNE CHARLES ET AL) 16 June 1998 (1998-06-16) column 4, line 8 - line 44 column 12, line 26 -column 13, line 14; claims 1-8; examples 35-39,47 --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 January 2000	04/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van Klompenburg, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 18869

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 7 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern .ıal Application No
PCT/US 99/18869

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5766597 A	16-06-1998	US 5494807 A	27-02-1996
		US 5756101 A	26-05-1998
		US 5364773 A	15-11-1994
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		CA 2105277 A	08-09-1992
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		EP 0575544 A	29-12-1993
		JP 6505397 T	23-06-1994
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WO 9633736 A	31-10-1996	AU 5851296 A	18-11-1996